Glycoacetylenes in the synthesis and application of stable glycoamino acids and peptides

Een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen, Wiskunde en Informatica

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List of abbreviations

Ac	acetyl
AFM	atomic force microscopy
Alloc	allyloxycarbonyl
aq.	aqueous
Ar	aryl
asc.	ascorbate
Bn	benzyl
Boc	*tert*-butoxycarbonyl
br	broad (NMR)
Cbz	carbobenzyloxy
CI	chemical ionization (MS)
CuAAC	Cu(I)-mediated azide-alkyne cycloaddition
d	days
d	doublet (NMR)
d.e.	diastereomeric excess
DCC	*N,N'*-dicyclohexylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DNP	dinitrophenyl
DSC	differential scanning calorimetry
e.e.	enantiomeric excess
e.g.	exempli gratia (for example)
EDCI	*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide
Endo A	*endo*-β-*N*-acetylglucosaminidase A
Endo M	*endo*-β-*N*-acetylglucosaminidase M
equiv.	equivalent(s)
ESI	electronspray ionization
et al.	*et alia* (and others)
Et	ethyl
FAB	fast atom bombardment (MS)
Fmoc	9-fluorenylmethoxycarbonyl
h	hour(s)
HAB	1,2,3,4,5,6-hexakis(azidomethyl)benzene
HBTU	*O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HGB	1,2,3,4,5,6-*hexakis*-(glucosyltriazolylmethyl) benzene
HIV	human immunodeficiency virus
HMPA	Hexamethylphosphoramide
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	*N*-hydroxybenzotriazole
HRMS	high resolution mass spectroscopy
i.e.	*id est* (that is)
J	coupling constant (NMR)
KHMDS	potassium hexamethyldisilazide
m	multiplet (NMR)
m/z	mass to charge ratio
MALDI	matrix-assisted laser desorption ionization
MBHA	*p*-methylbenzhydrylamine
Me	methyl
MGB	*mono*-(glucosyltriazolylmethyl) benzene
min	minute(s)
Ph	phenyl
PMDETA	*N,N,N',N'',N''*-pentamethyl diethylene triamine
PNGase F	Peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F
ppm	parts per million
q	quartet (NMR)
quant.	quantitatively
RCAM	ring-closing alkyne metathesis
R_f	retention factor
RNase B	ribonuclease B
rt	room temperature
s	singlet (NMR)
sat.	saturated
SEM	scanning electron microscopy
SPPS	solid phase peptide synthesis
Su	sucinimide

t	triplet (NMR)
TAB	1,3,5-tris(azidomethyl)-2,4,6-trimethylbenzene
TBDPS	*tert*-butyldiphenylsilyl
TBS	*tert*-butyldimethylsilyl
TBTA	*tris*-(benzyltriazolylmethyl)-amine
t-Bu	*tert*-butyl
TEM	tunneling electron microscopy
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
TGB	1,3,5-*tris*-(glucosyltriazolylmethyl) 2,4,6-methylbenzene
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
TMEDA	*N,N,N′,N′*-tetramethylethane-1,2-diamine
Tr	triazolyl

Amino acids

Ala	alanine (A)
Arg	arginine (R)
Asn	asparagine (N)
Asp	aspartate (D)
^{C}TGA	triazole-linked *C*-glycoamino acid
Cys	cysteine (C)
Dab	2,4-diaminobutanoic acid
Dap	2,3-diaminopropanoic acid
Gln	glutamine (Q)
Glu	glutamate (E)
Gly	glycine (G)
His	histidine (H)
Ile	isoleucine (I)
Leu	leucine (L)
Lys	lysine (K)
Met	methionine (M)
^{N}TGA	triazole-linked *N*-glycoamino acid
Orn	ornithine (O)
Phe	phenylalanine (F)
Pro	proline (P)
Ser	serine (S)
T1B	(1',2',3'-triazol-1'-yl)propylglycine
T1E	(1',2',3'-triazol-1'-yl)propylglycine
T1M	(1',2',3'-triazol-1'-yl)methylglycine
T1P	(1',2',3'-triazol-1'-yl)propylglycine
T4M	(1',2',3'-triazol-4'-yl)methylglycine
Thr	threonine (T)
Trp	tryptophane (W)
Tyr	tyrosine (Y)
Val	valine (V)

Carbohydrates

Gal	galactose
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
Lac	lactose
LacNAc	*N*-acetyllactoseamine
Man	mannose
Neu	neuraminic acid

Contents

1 From glycoacetylenes to glycoamino acids

Abstract

Glycoacetylenes, carbohydrate derivatives functionalized with an acetylene-function at one of the ring-carbons, can be synthesized by a range of different methodologies. In general, the methods leading to glycoacetylenes comprise the application of nucleophilic metal acetylide addition to carbohydrate electrophiles, in particular at the anomeric carbon. A sophisticated choice of conditions and metal counterions (*e.g.* magnesium, lithium, zinc and tin) gives selective formation of either α- or β-configured glycoacetylenes.

Glycoacetylenes can be applied in different ways for the synthesis of *C*-glycoamino acids. Examples given involve the synthesis of alkane-, alkyne-, tryptophan-, and triazole-linked glycoamino acids, as well as conformationally restricted glycoamino acids.

1.1 Synthesis of glycoacetylenes

C-Glycosidation is of great significance in the synthesis of optically active compounds, since it allows the introduction of carbon chains into sugar chirons.[1, 2] A *C*-glycoside results when the anomeric oxygen of a glycoside is replaced by a carbon atom (Figure 1.1). Versatile *C*-glycosidic building blocks are glycoacetylenes, which are carbohydrate derivatives functionalized with an acetylene at the anomeric center. This chapter will focus on the synthesis of the latter C-glycosides.

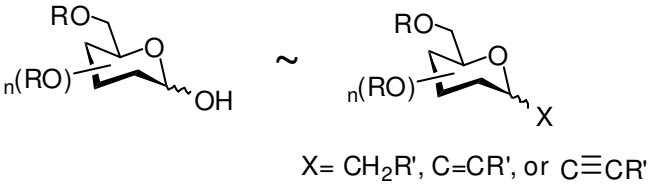
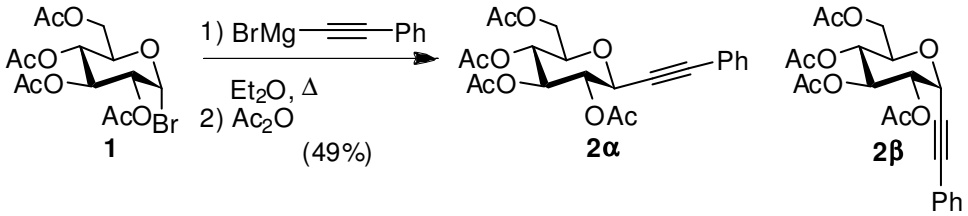


Figure 1.1 Replacement of the anomeric oxygen by a carbon results in a *C*-glycoside

Sugar-derived acetylenes are attractive due to the presence of a triple bond that can be applied as a handle for attachment to other chiral molecules or carbohydrate analogues. In addition, sugar-derived acetylenes are useful chiral scaffolds for the synthesis of natural products such as ciguatoxin[3, 4] and tautomycin,[2] or for the preparation of glycosidase inhibitors and lead compounds for drug discovery.[5]

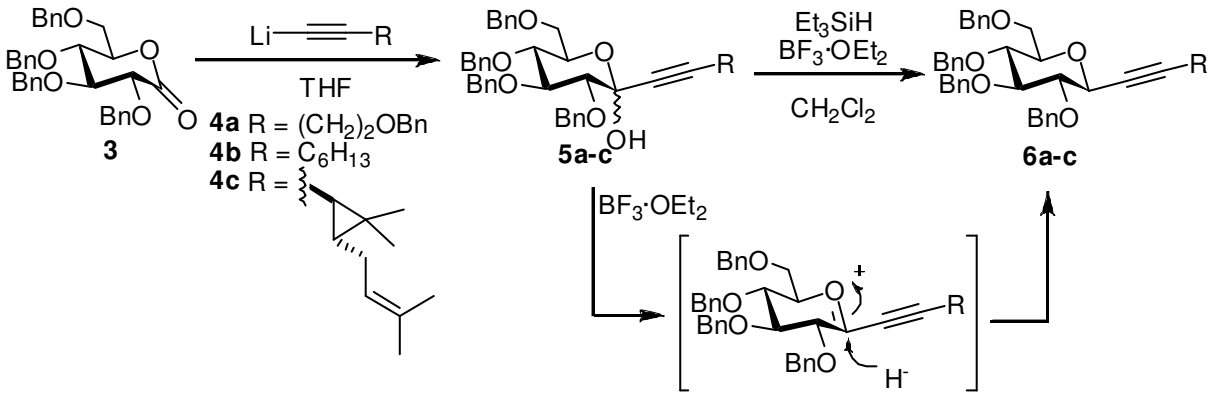
In 1958 the first example of a glycosylated acetylene was reported by Zelinsky and Meyer,[6] prepared by addition of phenylethynylmagnesium bromide to peracetylated α-D-glycopyranosyl bromide **1**, leading to a 3:1 mixture of β and α–configured glycoacetylenes **2** in 49% yield (Scheme 1.1).



Scheme 1.1 Addition of phenylethynylmagnesium bromide to tetraacetyl-α-D-glycopyranosyl bromide **1**

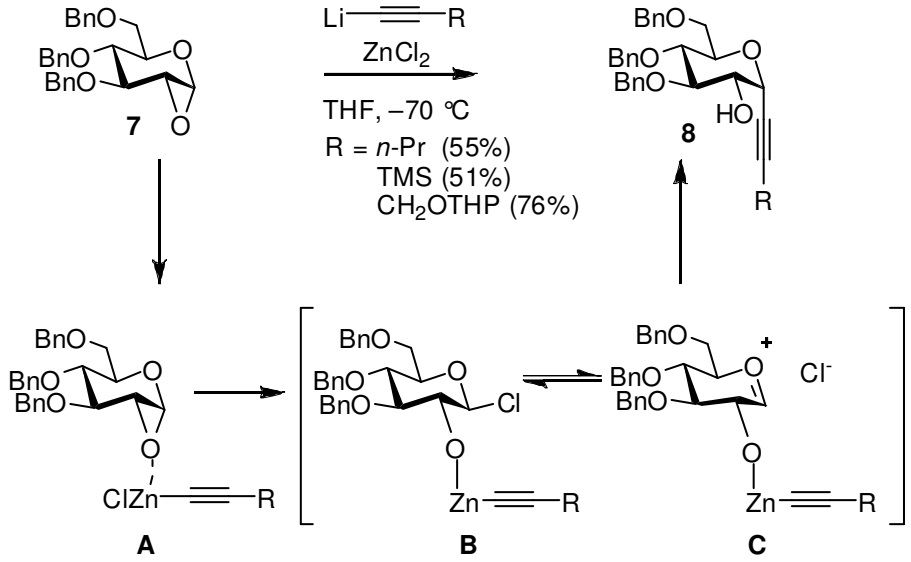
Decades later Sinaÿ *et al.*[7] achieved a fully stereoselective synthesis of *C*-β-D-glycopyranosylalkynes by the sequential addition of lithium acetylides to

gluconolactone **3**, followed by deoxygenation of the intermediate hemiacetal using triethylsilane and a Lewis acid. The axial attack of hydride to the pyranoid oxonium species generated by the Lewis acid proceeded with excellent stereochemical control (Scheme 1.2). From compound **4c**, a fragment of the antifungal antibiotic ambruticin was synthesized after selective reduction of the triple bond to *E* double bond and protective group removal.



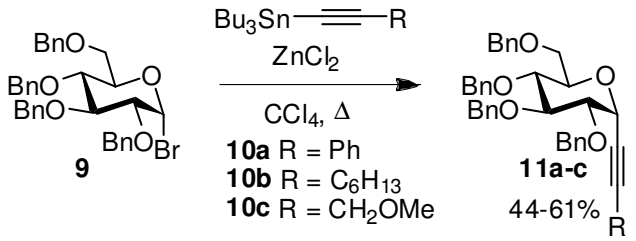
Scheme 1.2 Lithium acetylide addition to lactone **3**

The lithium acetylide-mediated synthesis of glycoacetylenes is by far the most common method for the synthesis of β-configured glycoacetylenes.[8] For the synthesis of α-glycoacetylenes, however, a different strategy must be applied. For example, lithium acetylide addition onto the readily obtained α-1,2-epoxide **7**,[9] induced by the weak Lewis acid zinc chloride, leads stereoselectively to α-glycoacetylene **8** (Scheme 1.3).[10] The selectivity in the addition can be explained by formation of a zinc acetylide intermediate that chelates to the epoxide to form the anomeric β-chloride alkoxy-zinc complex **B**, which is in equilibrium with the ion-pair **C**. Intramolecular α-directed delivery of the alkynyl moiety results in the formation of α-*C*-glycoside **8**.[11]



Scheme 1.3 Zinc-mediated lithium acetylide addition to α-1,2-epoxide **7**

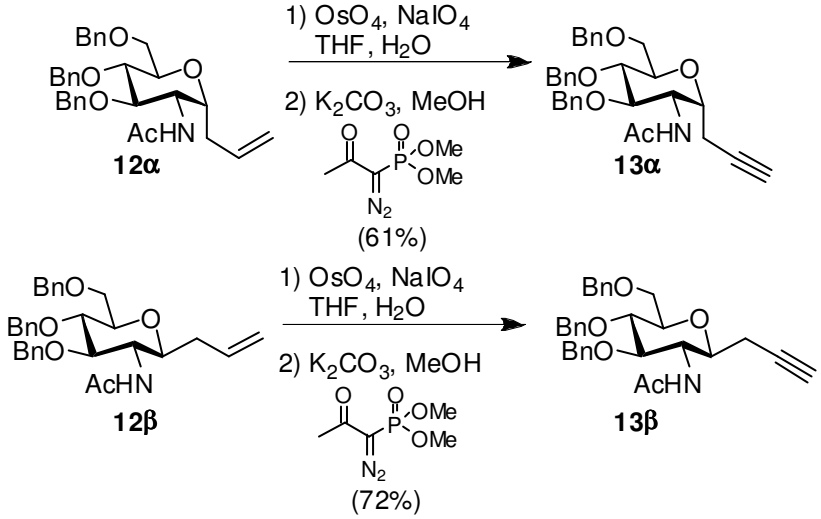
In 1988, Williams *et al.* reported the alkynylation of mixed acetals with organotin acetylides **10**.[12] Addition of such organotin acetylides to glycopyranosyl bromide **9** in the presence of zinc chloride, gave the desired alkynyl glycopyranose **11** with good α-selectivity in a moderate yield of 44-61% (Scheme 1.4). Similar addition reactions have been reported under the action of a variety of Lewis acids, such as silver tetrafluoroborate at low temperature (−30-0 °C),[13] or TMSOTf.[8, 14] The latter method is, in contrast to the acetylide addition onto epoxides, not only more widely applicable, but can also be used for the synthesis of glycosamines with an azide at C-2.[8]



Scheme 1.4 Addition of organotin acetylides to glucopyranosyl bromide **9**

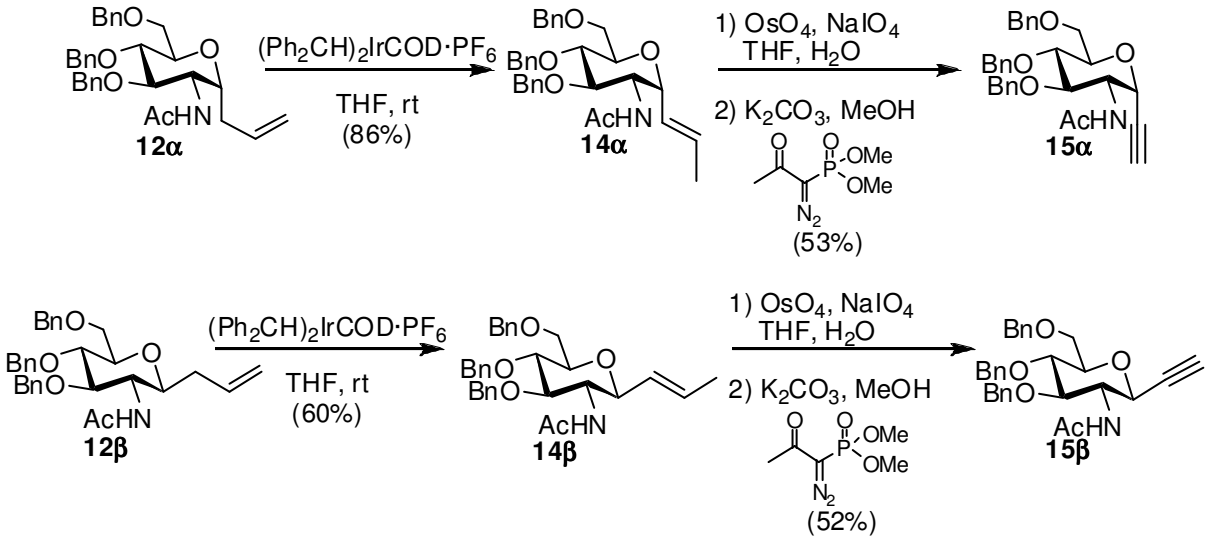
McGarvey *et al.*[15] developed an alternative route involving oxidative double bond cleavage[16] of allylic glycosides **12**, readily prepared by allyltin addition to the corresponding chloride in the presence of AIBN,[17, 18] followed by Seyferth-Gilbert homologation with the Bestmann-Ohira reagent to give propargylated glycosides **13**

(Scheme 1.5). Though such a route is not straightforward, it provides a suitable solution to the lack of direct stereoselective propargylation methodology.[19]



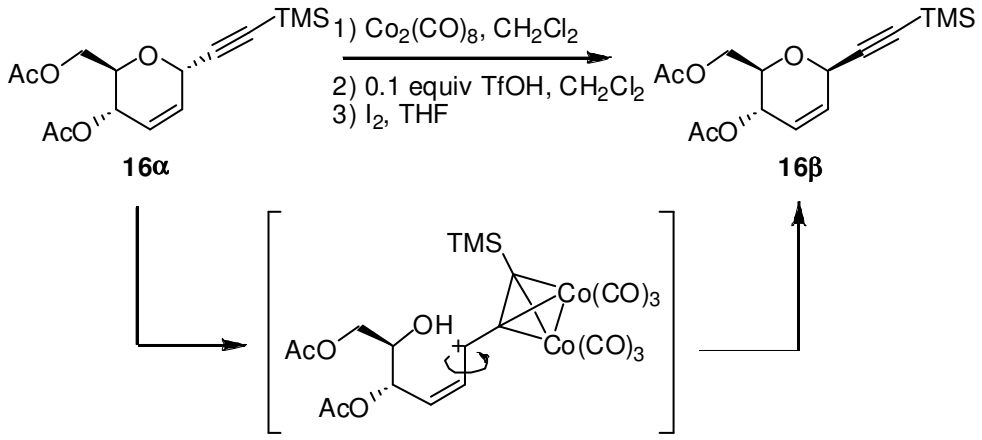
Scheme 1.5 Synthesis of propargyl glycosides

Apart from the oxidation-chain extension methodology presented above, metal-catalyzed isomerization of the intermediate allylic glycosides was examined (Scheme 1.6), thereby providing access to the glycoacetylenes **15** via the same sequence of conversions. Also by this strategy both α- and β-configured alkynyl glycosides can be obtained.



Scheme 1.6 Isomerization of allylic glycosides and conversion to glycoacetylenes

In some specific cases, an epimerization strategy can also be applied to convert α-configured acetylenes into β-glycosides (Scheme 1.7).[3,10,13] Thus, reaction of glycoacetylene **16α** with dicobalt octacarbonyl leads to the formation of the corresponding acetylene-dicobalt hexacarbonyl complex. The latter complex, upon treatment with an acid such as TfOH, generated a propargyl cation as stable as the triphenylmethyl cation. Consequently, the α-configured alkyne can be readily epimerized to the more stable β-configuration via the oxocarbenium ion. The α/β ratio (*i.e.* 1:6) is constant in equilibrium at 40 °C.



Scheme 1.7 Epimerization from α-linked **16α** to β-glycoside **16β**

1.2 Synthesis of *C*-glycoamino acids

C-glycosides are often applied as isosteres of natural glycosides in the synthesis of glycopeptides. Natural glycopeptides *i.e. O*- and *N*-glycopeptides, are rather labile with respect to chemical and enzymatic degradation.[20,21] *C*-Glycopeptides are therefore interesting isosteres, because of the high stability of the *C*-glycosidic bond against chemical and enzymatic cleavage (Figure 1.2).[5]

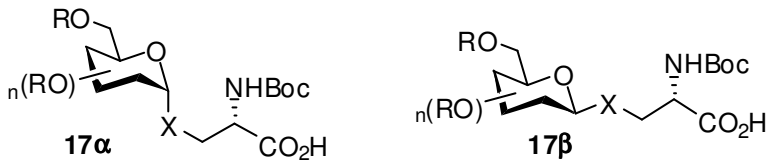
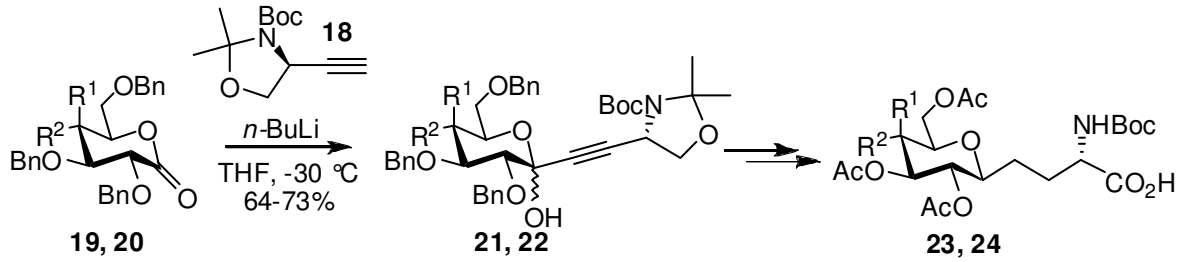


Figure 1.2 Natural α- and β-linked *O*-glycosyl serines (X = O) and the corresponding methylene isosteres (X = CH_2).

Dondoni *et al.*[5] reported a new expeditious method for the synthesis of β-linked *C*-glycosyl serines employing the ethynyloxazolidine **18** as a homoalanine precursor and sugar lactones as glycosidic electrophiles. Thus, acetylene **18**[22] was converted with *n*-BuLi in THF into the corresponding acetylide thereby leaving the configuration of the stereocenter of the oxazolidine ring intact (Scheme 1.8). A slight excess (1.3 equiv) of the lithium acetylide was added to D-galactonolactone **19** in THF at −30 °C, to afford a mixture of diastereomeric oxazolidinylacetylenes **21** in 64% isolated yield. Applying the same conditions to gluconolactone led to the isolation of **22** in a yield of 73%. The derivatives **21** and **22** were converted into the desired β-D-linked *C*-glycosyl serines **23** and **24** in 4 steps.[5]



Scheme 1.8 *Galacto*-series: for **19, 21** R^1 = OBn, R^2 = H; for **23** R^1 = OAc, R^2 = H. *Gluco*-series: for **20, 22** R^1 = H, R^2 = OBn; for **24** R^1 = H, R^2 = OAc.

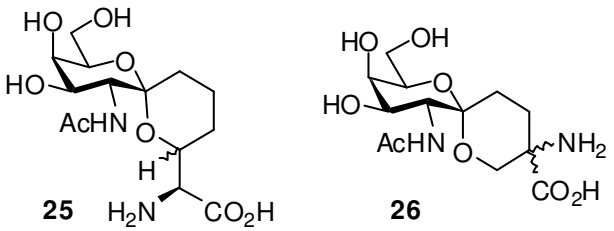
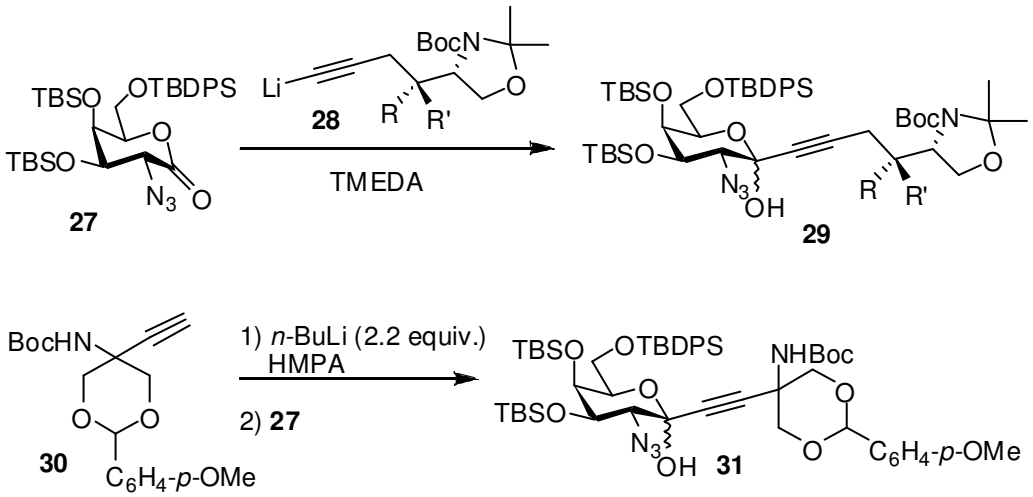


Figure 1.3 Conformationally constrained glycosylated amino acids

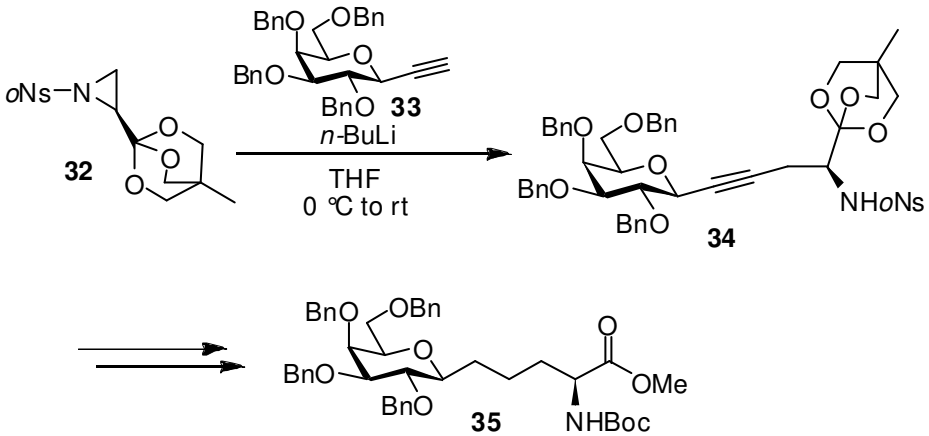
In the synthesis of conformationally constrained glycoamino acids **25** and **26** (Figure 1.3), Halcomb *et al.*[23-25] describes similar lithium acetylide additions on sugar-derived lactones, with the difference that oxazolidine **28** and acetal **30** were applied as the amino acid precursors and addition is performed in the presence of TMEDA[23] or HMPA[24] (Scheme 1.9). The latter additives have a dual function to avoid removal of the silyl protective groups, as well as to make the acetylides more nucleophilic, since in the absence of TMEDA and HMPA no addition of the acetylide to lactone **27** occurred. Both compounds **29** and **31** were obtained mixtures of anomers, although

the ratio could not be determined. Precursors **29** and **31** could be converted in several steps to **25** and **26**, respectively.



Scheme 1.9 Lithium acetylide addition to lactone **27**

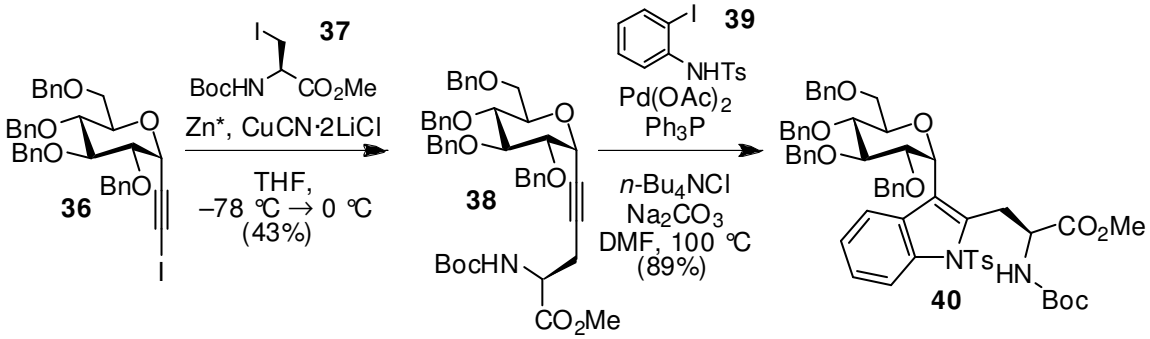
The perbenzylated β-D-galactopyranosyl-ethyne **33** was prepared by van Boom *et al.*[26] based on a procedure of Meldal *et al.*[27] From the nosyl-protected aziridine orthoester **32**, *C*-glycosidic amino acid **35** was prepared by ethynylation with the acetylide derivative of compound **33** to give the ring-opened product **34** in a yield of 82% (Scheme 1.10).



Scheme 1.10 Lithium acetylide addition to aziridine **32** provides a direct precursor to glycoamino acid **35**

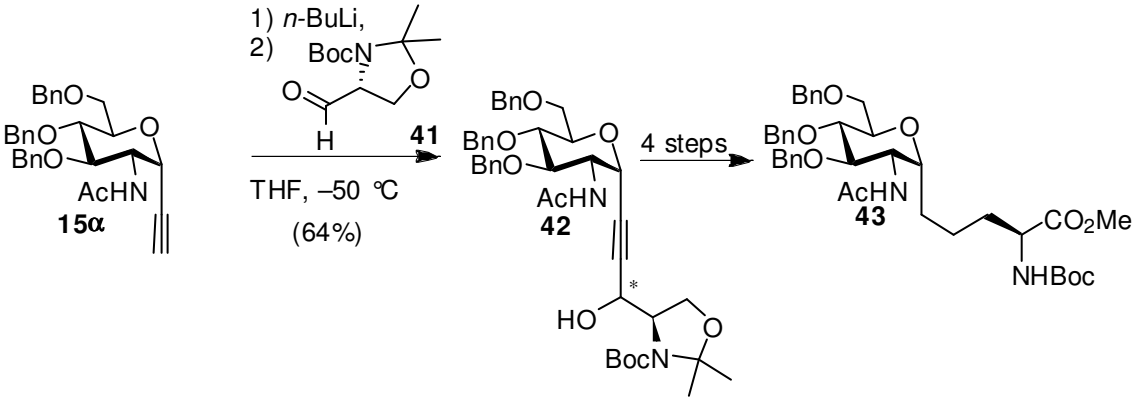
Our group investigated organozinc couplings of amino acids to carbohydrates,[28,29] involving alkyl iodide derivatives of amino acids and iodo- or bromoacetylene

derivatives of the carbohydrate. In the presence of Rieke zinc, insertion in the iodine-carbon bond takes place, followed by trans-metallation exchange by copper(I). The resulting zinc/copper species reacts with the halogenated acetylene to form the desired carbon-carbon bond. For example, application of the method to bromoglucoacetylene **36** and iodoalanine **37** gave the acetylene-linked glycoamino acid **38** in moderate yield.[29] Around the same time, Isobe *et al.*[30] published a similar reaction (Scheme 1.11) and used the thus formed glucoamino acid **38** for further transformation to α-*C*-glycosyltryptophan **40** using Larock's palladium-catalyzed heteroannulation methodology.[31] Interestingly only *iso*-tryptophan derivative **40** was formed instead of the expected tryptophan.

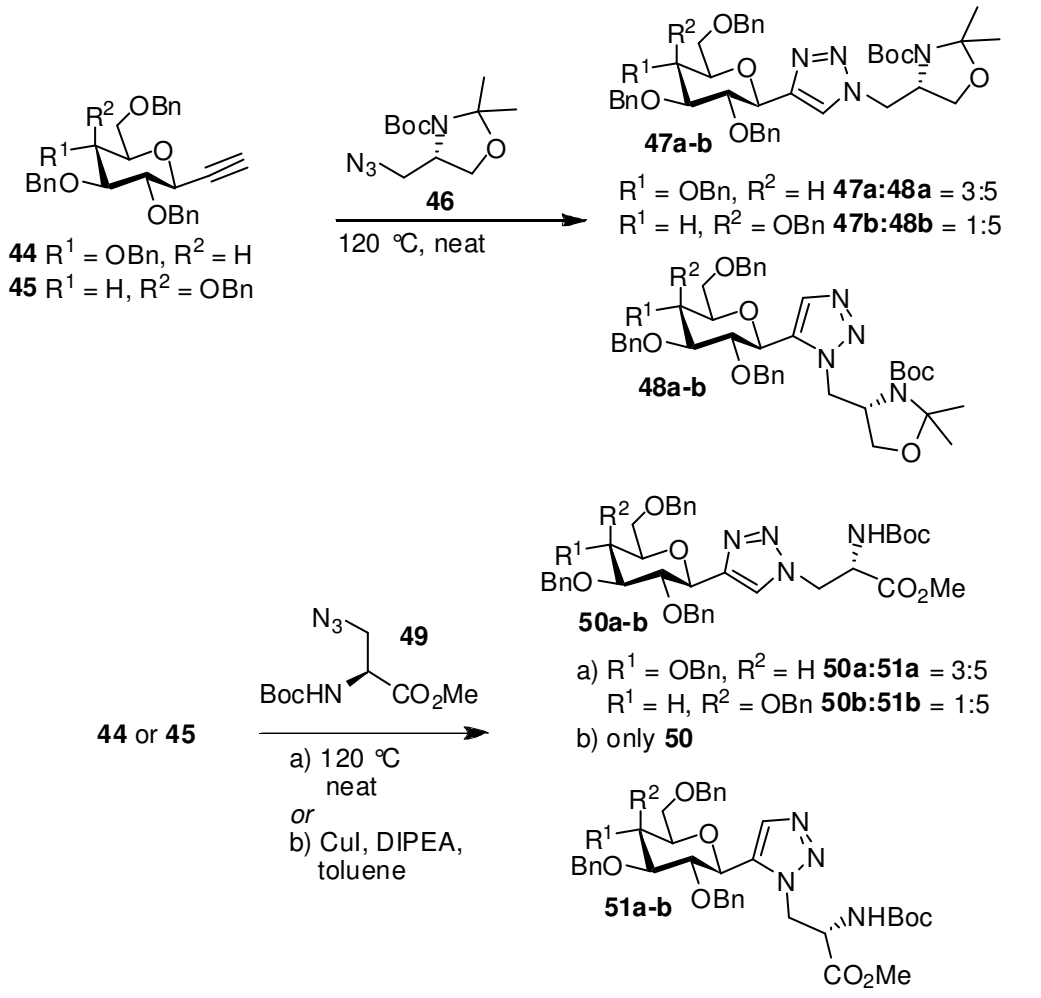


Scheme 1.11 Organozinc coupling of acetylene **36** and iodoalanine **37** and the formation of tryptophan-linked glycoamino acid **40**

The α- and β-glycoacetylenes described in the previous paragraph were also applied by Dondoni *et al.* in the synthesis of glycoamino acids involving Li-acetylide addition to Garner's aldehyde **41**.[8, 32] An example of this addition is shown in Scheme 1.12, which is followed by the subsequent transformation to the desired glycoamino acid **43**, via hydrogenation, deoxygenation, and oxidative cleavage of the oxazolidine ring. Following this protocol, a set of 12 different *C*-glycosyl asparagine isosteres, having the amide linkage replaced by an ethylene and six pairs of α- and β-anomers, were prepared incorporating gluco-, galacto-, manno- and the corresponding 2-acetamido-2-deoxygluco-residues.



Scheme 1.12 Lithium acetylide addition to Garner's aldehyde **41**



Scheme 1.13 1,3-Dipolar cycloaddition by Dondoni *et al.*

In 2004, Dondoni[33] and our group[34] independently reported the synthesis of triazole-linked glycoamino acids from glycoacetylenes and azidoamino acids, as described extensively in Chapter 3. Dondoni initially studied the 1,3-dipolar cycloadditions by heating the glycoacetylenes **44** and **45** and azidomethylene-oxazolidine **46** without solvent (Scheme 1.13). Under these conditions, two regioisomers were formed, *i.e.* the 1,4- and 1,5-substituted triazoles with a preference for the 1,5-regioisomer **48**. Cycloaddition to azidoalanine **49** afforded similar regioisomer ratios (conditions a). In contrast, the newly developed copper(I)-catalyzed cycloaddition methodology (condition b)[35, 36] proved highly fruitful, producing only the 1,4-regioisomer **50**.

1.3 Purpose and outline of this thesis

In this thesis, the development of new methods to synthesize stable *C*-glycoamino acids from glycoacetylenes is investigated. The resulting glycoamino acid isosteres are incorporated into glycopeptide structures by chemical and enzymatic procedures and compared to the natural glycoamino acids in order to evaluate the value of the triazole function as an amide isostere. Apart from that, the glycoacetylenes are applied in the preparation of two oligomeric glycosides of which the supramolecular behavior was studied.

Chapter 1 gives an overview of the synthesis of glycoacetylenes and their application in the synthesis of stable *C*-glycoamino acids.

In Chapter 2 ring-closing alkyne metathesis (RCAM) is presented as a new tool to synthesize alkyne-linked glycoamino acids.

The synthesis of triazole-linked glycoamino acids and glycopeptides via copper(I)-catalyzed azide–alkyne cycloaddition is described in Chapter 3.

Chapter 4 delineates the chemoenzymatic transglycosylation of triazole-linked *C*- and *N*-glycopeptides. Furthermore the resistance towards PNGase digestion of a triazole-linked glycopeptide is shown.

Chapter 5 details the synthesis of oligomeric glucosides via copper(I)-catalyzed azide–alkyne cycloaddition. The supramolecular aggregation of these compounds is studied.

Chapter 6 describes *alcalase*-mediated enzymatic peptide coupling involving the incorporation of nonproteinogenic amino acids, i.e. alkyne-, azide-, and triazole-linked glycoamino acids.

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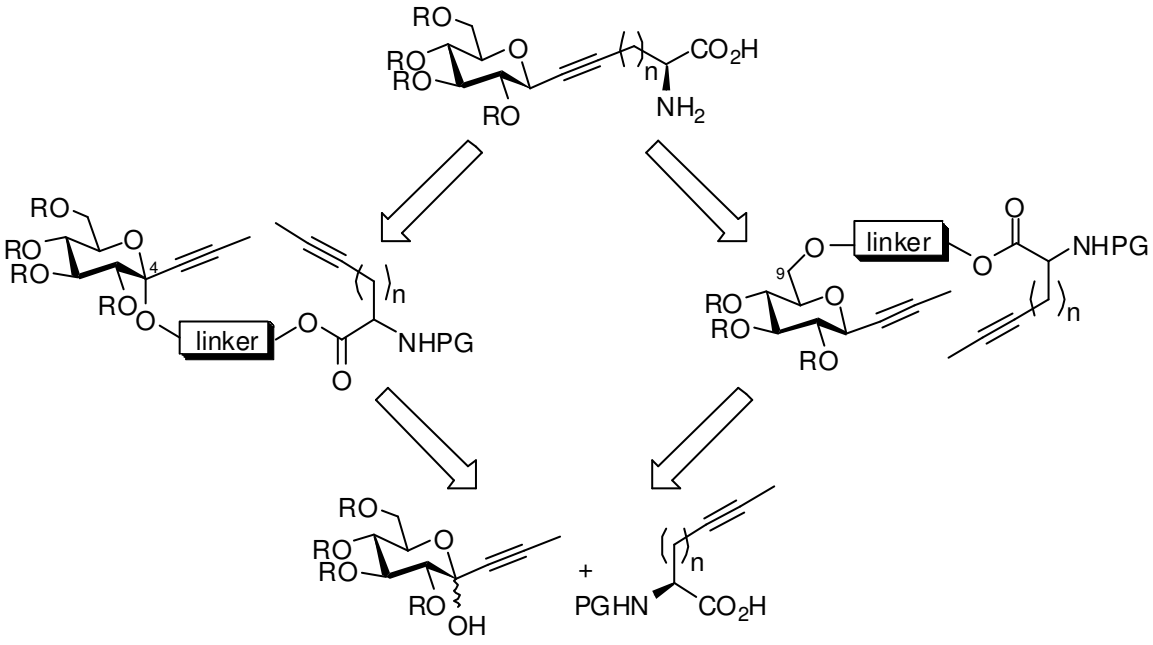
2 Ring-closing alkyne metathesis in the synthesis of alkyne-linked glycoamino acids

Abstract

Synthesis of alkyne-linked glycoamino acids via ring-closing alkyne metathesis is investigated. Two different strategies are described to attach alkynylamino acids to an alkynylsugar, via a linker at O-4 or O-9 of the alkynylsugar. Subsequent ring-closing alkyne metathesis of the O-4–linked diynes failed to proceed, but alkyne-linked glycoamino acids of different chain length were effectively synthesized via attachment at O-9.

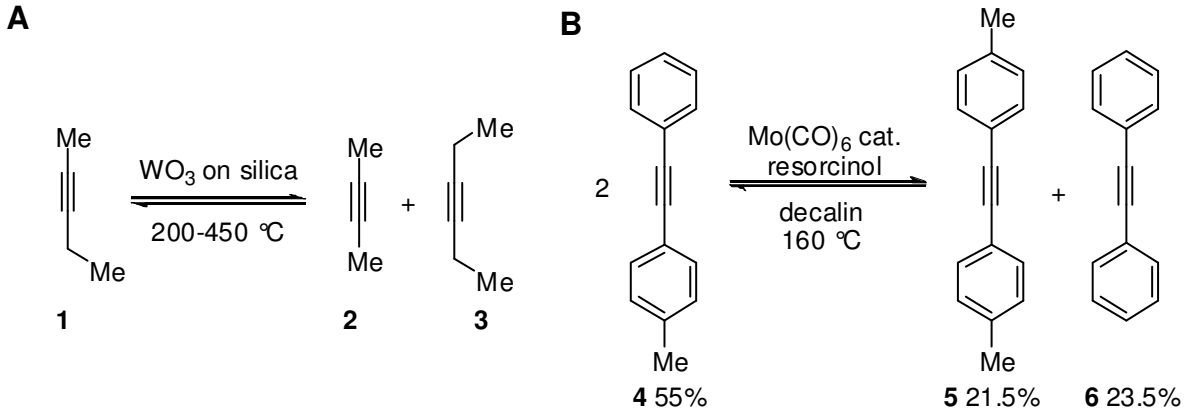


Part of this chapter has been published: Groothuys, S.; van den Broek, S.A.M.W.; Kuijpers, B.H.M.; IJsselstijn, M.; van Delft, F.L.; Rutjes, F.P.J.T., *Synlett* **2008**, 111.

2.1 Introduction

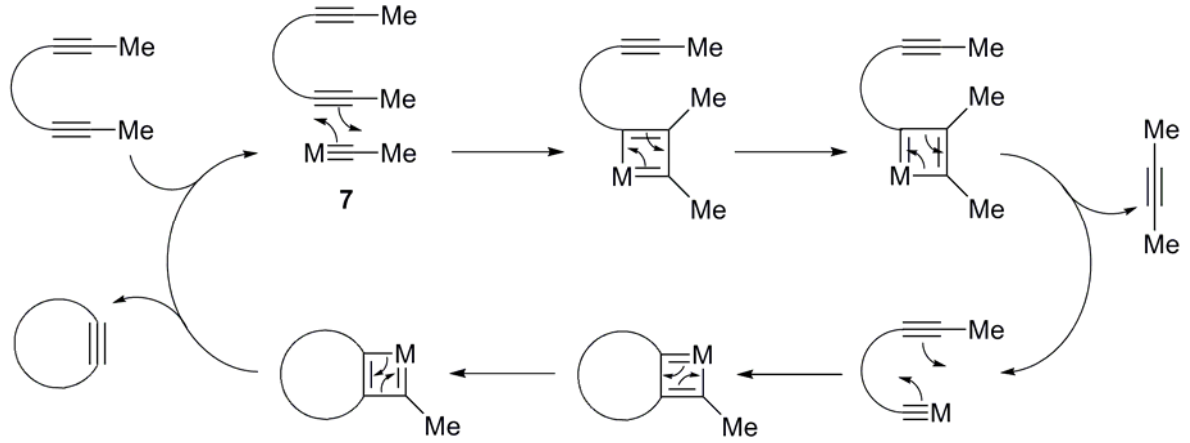
Rarely a novel synthetic transformation attained wide application in synthetic organic chemistry as rapidly as the olefin metathesis reaction for the synthesis of unsaturated carbocycles. Although already broadly applied in hydrocarbon industry, not until the early 1990s olefin metathesis attained legendary status in synthetic chemistry, largely owing to the preparation of the first efficient well-defined catalyst by Schrock,[1] and to the efforts of Grubbs, who developed air stable catalysts with much broader functional group compatibility.[2] Not surprisingly, the Nobel Prize in chemistry was awarded to Grubbs and Schrock in 2005, as well as to Chauvin, who already in 1971 proposed the actual mechanism of olefin metathesis and predicted what types of metal compound could act as catalysts.[3] The number of applications of olefin metathesis in the mid 1990s rose steeply and at present forms an indispensable technique in the organic chemistry toolbox.

A conceptually similar transformation is alkyne metathesis. As indicated by the name alkyne metathesis, first reported by Pennella *et al.* in the late 1960s,[4] and applied in the synthesis of organic macrocycles by Fürstner *et al.*,[5] involves an exchange reaction of alkynes. The inherent advantages of alkyne over alkene metathesis reside in the fact that ring-closing alkyne metathesis (RCAM) is particularly useful for the synthesis of large (>12-membered) rings without high dilution and cannot lead to *E*,*Z*-mixtures. More than that, under the proper conditions the resulting triple bond can be stereoselectively reduced to either *E*- or *Z*-olefin. However, alkyne metathesis is lagging far behind olefin metathesis in popularity, particularly due to the lack of a mild catalyst not requiring stringent exclusion of moist or oxygen during reaction. Nevertheless large improvements have been achieved. Initially requiring temperatures as high as 400 °C when catalyzed by heterogeneous silica-bound tungsten oxides, Mortreux and coworkers later discovered[6] a soluble $Mo(CO)_6$ alkyne metathesis catalyst able to perform at much lower temperatures (Scheme 2.1).



Scheme 2.1 Alkyne metathesis by Pennella *et al.* (A) and Mortreux *et al.* (B)

Similar to olefin metathesis,[7] Katz postulated a mechanism for alkyne metathesis involving the metal alkylidyne complex **7**, and a sequence of formal [2+2] cycloaddition and cycloreversion steps as indicated for RCAM of a diyne in Scheme 2.2.[8]



Scheme 2.2 Mechanism of RCAM based on the proposed alkyne metathesis mechanism

The mechanism proposed by Katz has been experimentally corroborated by Schrock *et al.*[9] following the preparation of the well-defined tungsten catalyst [(^{t}BuO)$_3$W≡CCMe$_3$] (**8**) and demonstration of its applicability in the cross-metathesis of 3-heptyne.[10] Several intermediate complexes formed by the [2+2] cycloaddition were isolated and characterized. Apart from that, it was observed that only internal acetylenes undergo metathesis, with terminal acetylenes leading exclusively to polymerization.[11] Fürstner *et al.* developed several molybdenum catalysts of the general type [Mo{N(^{t}Bu)(Ar)}$_3$].[12] However, as mentioned, a major drawback of these

complexes is the incompatibility with oxygen, nitrogen, moisture, acidic protons, and even secondary amides.

In the last decade, several examples on the application of RCAM for the total synthesis of natural products were published, either in combination with partial selective reduction of the triple bond to the *Z*-double bond as in nakadomarin A,[13] sophorolipid lactone,[14] and lantrunculin B[15] (Figure 2.1) or in combination with other post-metathesis transformations, as for citreofuran.[16]

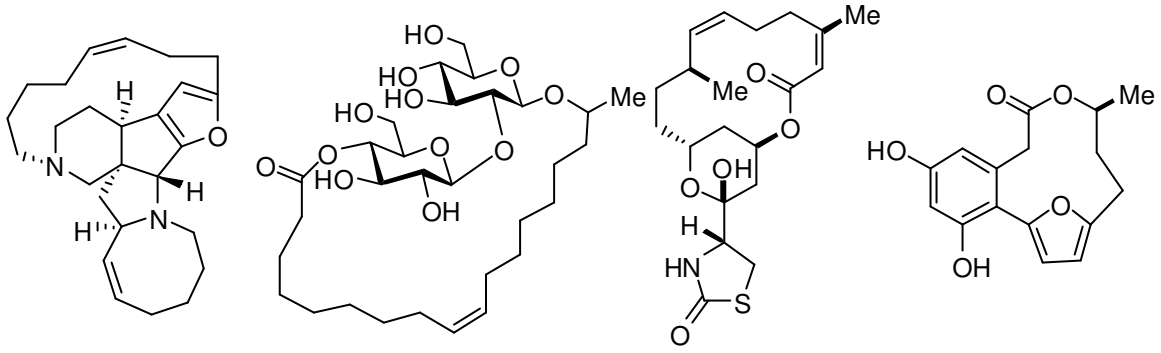
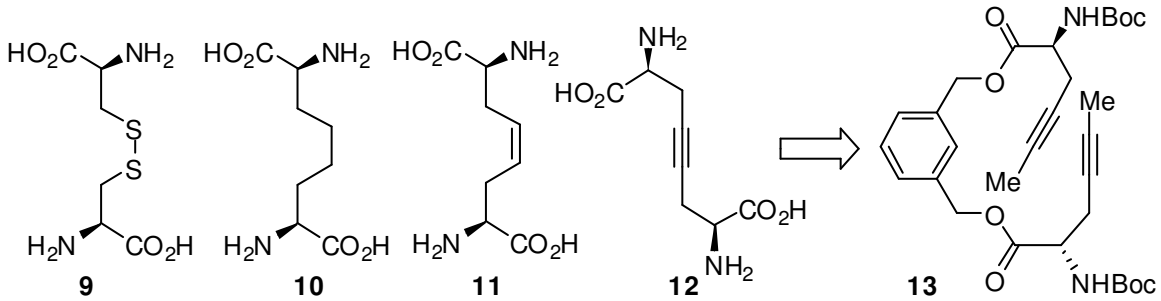
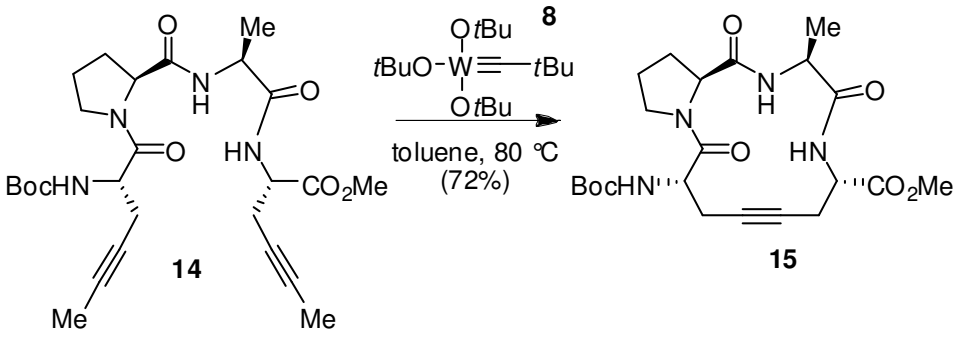


Figure 2.1 Natural products synthesized via RCAM



Scheme 2.3 Diaminosuberic acid (**9**) and derivatives (**10-12**) by Aguilera *et al.*[17]

An example from our own group in a collaborative effort with van Boom *et al.* shows that RCAM can be successfully applied in the synthesis of diaminosuberic acid derivatives **10-12**, each with an all-carbon chain mimicking the natural cystine bridge (**9**, Scheme 2.3).[17] Along the same line, we showed[18, 19] that conformationally restricted β-turn peptide mimetics can be obtained by performing RCAM on a dialkyne-containing linear peptide **14** as illustrated in Scheme 2.4, an approach followed as well by Liskamp *et al.*[20] in the synthesis of a mimic of the lantibiotic nisin Z.



Scheme 2.4 Conformationally restricted β-turn mimetics by IJsselstijn *et al.*[18]

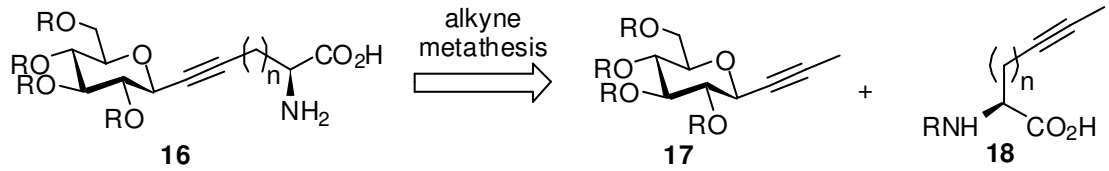
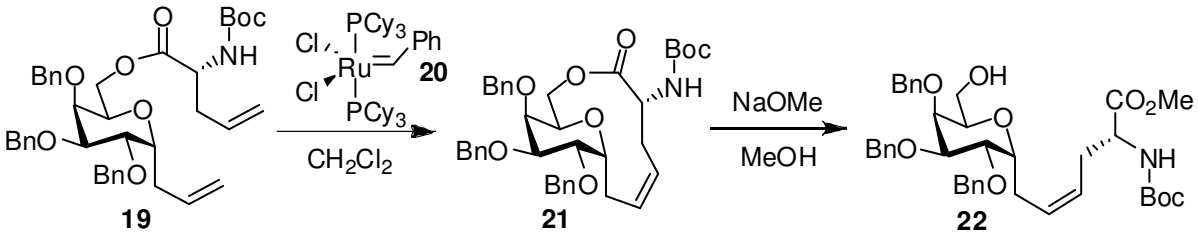


Figure 2.2 Retrosynthesis for alkyne-linked glycoamino acids



Scheme 2.5 Synthesis of alkene-linked glycoamino acids via RCM

The examples above demonstrate the versatility of RCAM as an alternative for olefin metathesis, in particular for the synthesis of cycloalkynes or *Z*-configured alkenes. Based on these observations, we became interested in the synthesis of alkyne-linked glycoamino acids via RCAM of alkynylglycoside **17** and 2-butynylglycine **18** (Figure 2.2). Not only is RCAM on carbohydrate derivatives largely unexplored, but the resulting alkyne-linked glycoamino acids **16** could also be valuable compounds for the assembly of chemically and metabolically stable all-carbon glycopeptide isosteres. Moreover, the alkyne-linked glycoamino acids can be chemo- and stereoselectively (partially) reduced, giving access to either the *E*- or the *Z*-configured alkene-linked derivatives from a common precursor.[21] Similar *C*-glycoamino acids **22** have earlier been prepared via olefin metathesis by Westermann *et al.*[22] (Scheme 2.5). However, with longer chains mixtures of *E*- and *Z*-cycloalkenes are formed upon ring closure.

2.2 RCAM of diynes linked at the anomeric center

Two retrosynthetic strategies were investigated for the assembly of alkyne-linked glycoamino acids **16** via RCAM of an alkynylsugar and 2-butynylglycine. One via connection to the carbohydrate at the former C-1 position, and the other via the former C-6 position (C-4 and C-9 in the alkynylsugar **16,** Figure 2.3).

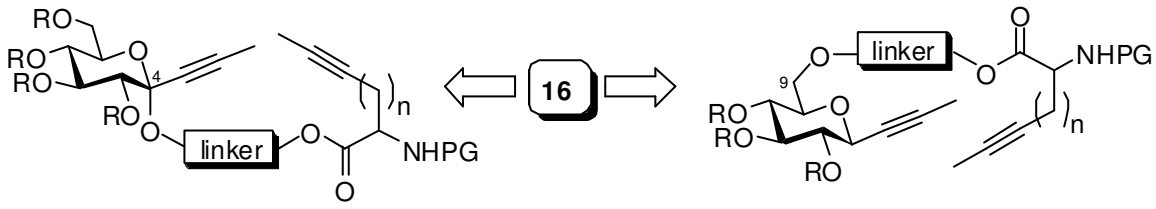
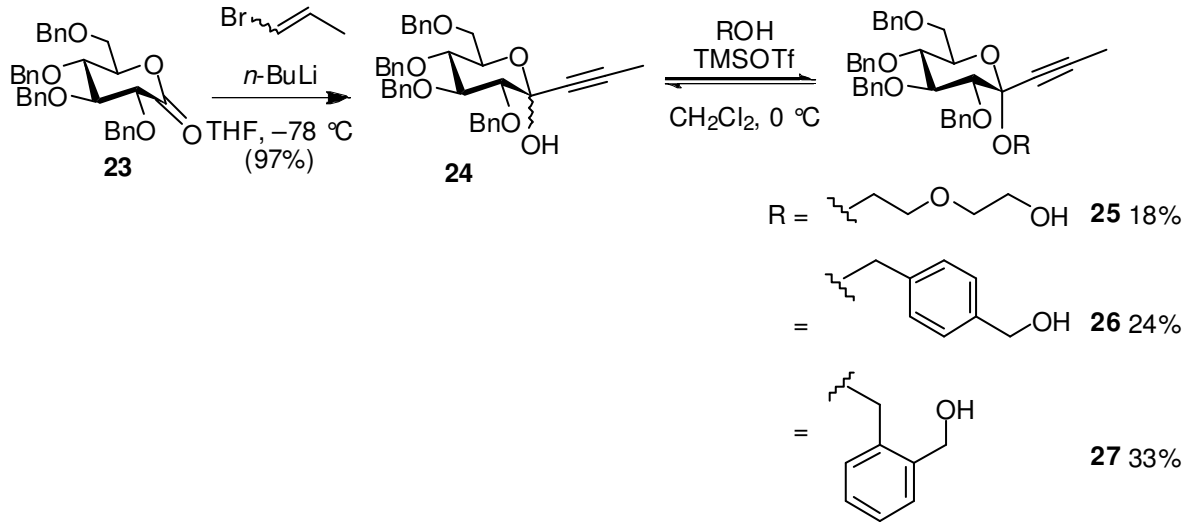


Figure 2.3 Retrosynthesis via C-4 and C-9 linkage

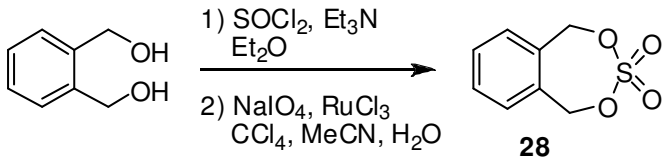
First, the RCAM route of diynes linked via C-4 was investigated. To this end, the known lactone **23,**[23] readily prepared from commercially available tetra-*O*-benzyl glucose by Swern-Moffat oxidation (DMSO, acetic anhydride), was converted into alkynylglucoside **24** (Scheme 2.6) by subjection to nucleophilic addition of Li-acetylide. The latter nucleophile was generated *in situ* from 1-bromopropene and 2 equivalents *n*-BuLi, similar to the procedure described by Leeuwenburgh *et al.*[24]



Scheme 2.6 Synthesis of anomerically coupled linkers

Since it is known that RCAM is suitable for the formation of cycloalkynes of ring sizes larger than 12,[13] a linker has to be introduced between the alkynylsugar and

2-butynylglycine. The newly generated anomeric center C-4, the former C-1 of gluconolactone, appeared ideally suitable for this purpose, in particular by attachment of a diol, thus leaving one alcohol free for esterification to 2-butynylglycine. Three different diols were investigated as linkers. Firstly, hemi-acetal **24** was condensed with diethyleneglycol, under the action of a Lewis acid (TMSOTf) in the presence of a drying agent ($MgSO_4$).[25] However, despite the fact that a large excess of diethyleneglycol was added (5 equiv), compound **25** could only be obtained in a disappointing yield of 18%, due to incomplete conversion and the formation of dimers. Apparently, an equilibrium is established between product and starting material, despite the presence of magnesium sulfate. Moreover, the five-fold excess of diethyleneglycol is apparently not sufficient to prevent double glycosylation with carbohydrate **24**. A similar result was obtained by addition of 1,4-benzenedimethanol instead of diethyleneglycol (**26**, 24%), while a slight increase in yield was observed for *ortho*-benzenedimethanol, particularly due to the isolation of smaller amounts of dimer, to afford the desired glycoside **27** in 33% yield.

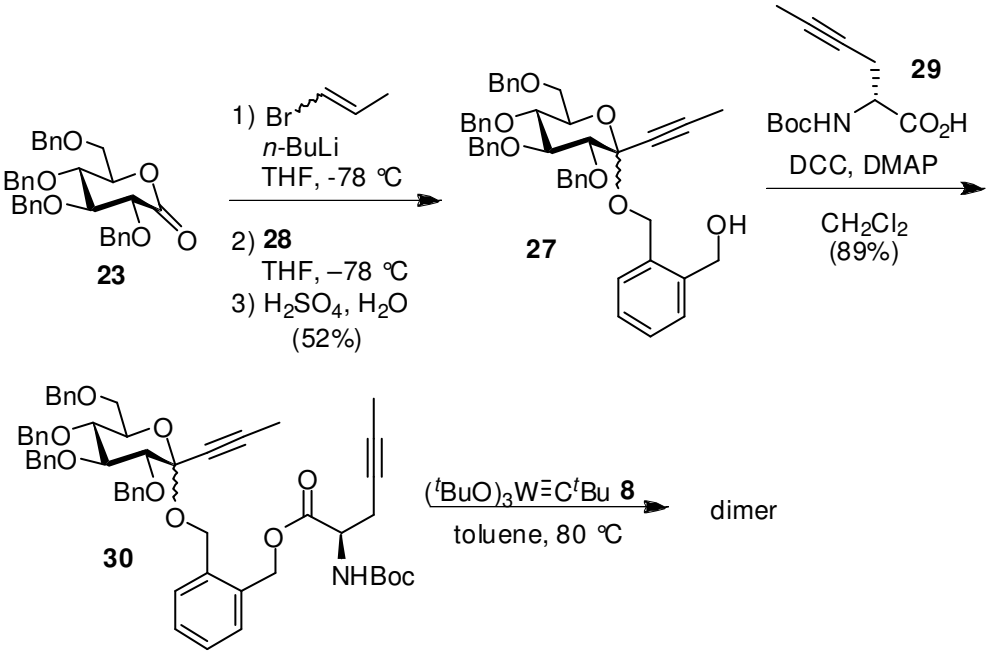


Scheme 2.7 Synthesis of cyclic sulfate **28**

An alternative mode of introduction of a linker, instead by dehydrative condensation with excess diol, entails nucleophilic attack of **24** at a suitable electrophile. Obviously, such a strategy could be attained by monoprotection of a diol, conversion to a suitable electrophile and condensation with **24**, before removal of the protective group. A more elegant approach involves the conversion of the diol to a cyclic sulfate, with the inherent advantage that only single addition is possible. Thus, as exemplified in Scheme 2.7, 1,2-benzenedimethanol was converted into cyclic sulfate **28**[26] as described by O'Brien *et al.*[27]

Moreover, it was realized that conversion of **24** into a good nucleophile could be effected by deprotonation with a suitable base (*e.g.* NaH), but a more direct approach would involve addition of cyclic sulfate **28** to the alkoxide formed *in situ* by acetylide addition to lactone **23** (Scheme 2.8). Subsequent removal of the *O*-sulfate with

sulfuric acid and water proceeds in a one-pot procedure leading to the desired alcohol **27** in 52% overall yield from lactone **23**. Compound **27** was isolated as an α,β-mixture in a ratio of 3:4.

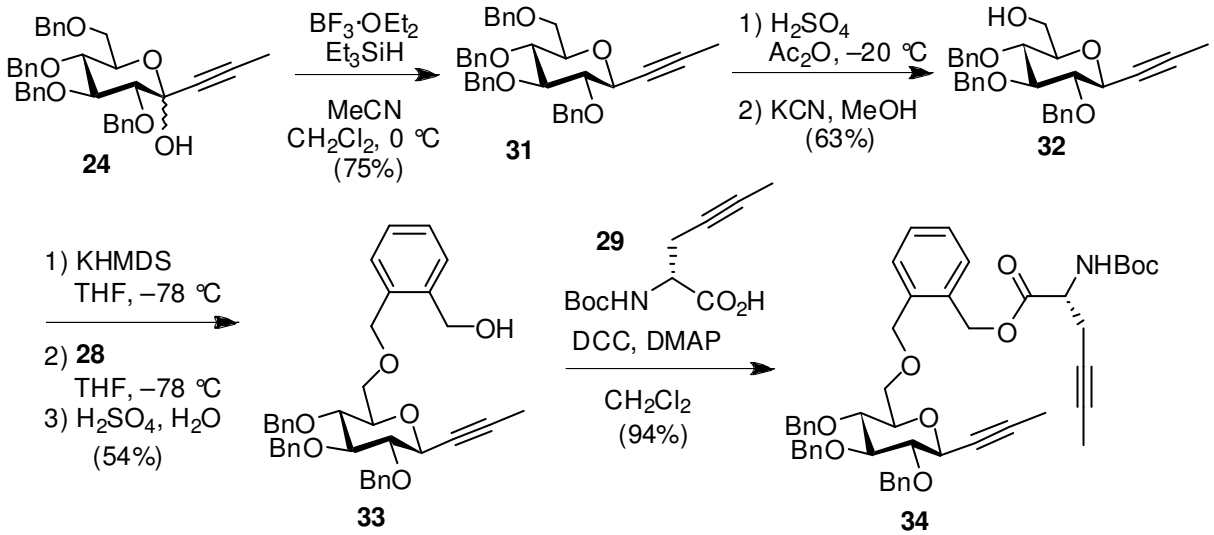


Scheme 2.8 Coupling of amino acid **29** to the anomeric linker and RCAM

Next, Boc-protected 2-butynylglycine **29**[28] was coupled to the free hydroxyl of **27** under the action of DCC and DMAP, leading to diyne **30** as the desired substrate for RCAM. Thus, compound **30** was dissolved in toluene and degassed with argon, before addition of the tungsten-based catalyst **8** in a glove box. Heating the reaction mixture to 80 °C for 30 minutes, followed by TLC analysis showed the disappearance of starting material and the formation of less lipophilic product. Unfortunately, analysis of the formed product after work-up and silica gel purification showed that solely cross-metathesis had occurred, leading to quantitative dimer formation.[29] Since the ring-size of the desired product (12-ring) is sufficiently large to enable formation of a cycloalkyne (as indicated by Fürstner's rule), a possible alternative explanation for the lack of ring-closure may be found in the fact that the tertiary anomeric center, directly adjacent to the sugar alkyne, causes too much steric hindrance.

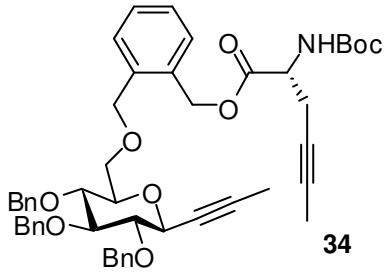
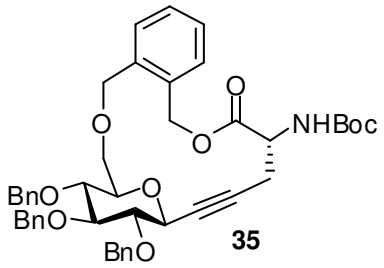
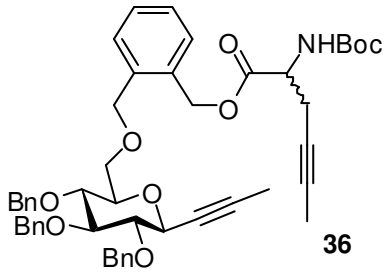
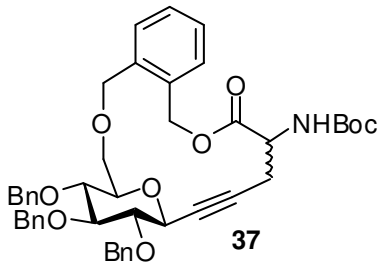
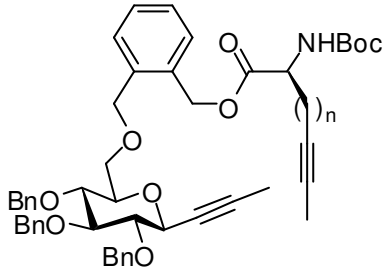
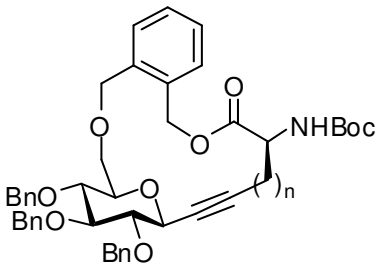
2.3 RCAM of diynes linked at the primary hydroxyl

Since the RCAM of a diyne linked via the anomeric center was not successful, another strategy was considered to attach 2-butynylglycine to the carbohydrate. Although in principle any other position in the carbohydrate could be applied, the C-9 position (former C-6) was considered most amenable for selective deprotection. An additional advantage of such a strategy is that a late-stage deoxygenation of the anomeric carbon (C-4) is avoided. Thus, the hemi-acetal at C-4 of **24** was first deoxygenated under the action of $BF_3 \cdot OEt_2$ and Et_3SiH yielding stereoselectively the β-configured alkynylglycoside **31** (Scheme 2.9).[30] Selective removal of the benzyl protecting group at O-9 by acetolysis with H_2SO_4 in acetic anhydride, followed by removal of the resulting acetyl group gave the desired glycoacetylene **32** with a free primary hydroxyl. Introduction of the linker could be performed under similar conditions as above. Thus, deprotonation of the alcohol by KHMDS in THF, and subsequent addition of cyclic sulfate **28** led to the chain-extended compound **33**. Diimide-mediated esterification of **33** with Boc-protected D-2-butynylglycine proceeded uneventfully, leading to the RCAM precursor **34** in excellent yield.



Scheme 2.9 Synthesis of gluco amino acid **34**

Table 2.1 Synthesis of RCAM precursors and RCAM products

entry	esterification[a]	yield	RCAM[b]	yield
1	 **34**	94%	 **35**	80%
2	 **36**	92%	 **37**	80%
3	 **3**	**8** (n = 2) 92%		**39** (n = 2) 62%
4		**40** (n = 3) 84%		**41** (n = 3) 29%

[a] DCC, DMAP, CH_2Cl_2; [b] **8**, toluene, 80°C

Diyne **34** was now subjected to identical conditions for ring-closing alkyne metathesis as described earlier. Thus, thorough drying and degassing was executed to avoid premature decomposition of the sensitive catalyst. Much to our satisfaction, prolonged treatment of **34** with the tungsten-based RCAM catalyst **8** led to the cyclized alkyne **35** in 80% yield (Entry 1, Table 2.1). The structure of cycloalkyne **35** was corroborated by NMR and mass spectral analysis. Since the formed glycoamino acid posessed the unnatural D-configuration at the amino acid α-carbon, we were interested if RCAM could also afford the more versatile L–isomer. To this end, due to the lack (at the time) of L–2-butynylglycine, racemic butynylglycine (±)-**29** was attached to the carbohydrate derivative **33** and subjected to ring–closure. No difference in reaction rate between the two epimers could be observed, leading to a

1:1 mixture of diastereomeric cycloalkynes **37**. Some L-diastereomeric glycoamino acids could in fact be prepared by applying L-3-pentynylglycine and L-4-hexynylglycine[28] to the synthetic sequence of esterification-RCAM (entries 3 and 4, respectively). In both cases, the desired cycloalkynes were successfully formed, although a clear negative correlation was observed between expansion of the amino acid side–chain, giving the corresponding cyclic acetylenes from 3-pentynylglycine and 4-hexynylglycine in yields of 62 and 29%, respectively. The RCAM of **40** gave only 0.8 mg and therefore the product could only be confirmed by HRMS.

2.4 Conclusions

Acetylene-linked glycoamino acids were successfully prepared by application of ring-closing alkyne metathesis. To this end, alkynylsugars and alkynylamino acids were coupled via a benzenedimethanol linker, derived from cyclic sulfate **28**. Attachment points for the amino acid were either the newly formed anomeric center at C-4 or the primary hydroxyl at C-9. Performing RCAM on the anomerically-linked dialkyne led exclusively to dimerization and failed to yield cycloalkyne product, presumably due to sterical hindrance. Linkage via O-9 proceeded smoothly for 2-butynylglycine derivatives of different configuration and chain-lengths, upon RCAM successfully leading to the desired glucoamino acids with ring-sizes varying between 15 and 17.

2.5 Acknowledgements

Bas van den Broek is gratefully acknowledged for a substantial contribution to this chapter. Dr. Maarten IJsselstijn is gratefully acknowledged for the synthesis of the tungsten catalyst, and experimental guidance during the RCAM.

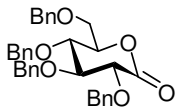
2.6 Experimental section

General information

^{1}H-NMR spectra were recorded on a Varian Inova 400 (400 MHz) spectrometer and ^{13}C-NMR spectra on a Bruker DMX300 (75 MHz) or Bruker DPX200 (50 MHz) spectrometer. Chemical shifts (δ) are given in ppm, downfield from tetramethylsilane. IR spectra were recorded on an ATI Mattson, Genesis series, or a Bruker Tensor 27 FTIR spectrometer. High resolution mass spectra were recorded on a JEOL AccuTOF (ESI), or a MAT900 (EI, CI, and ESI). All reactions were performed

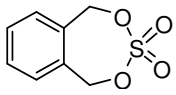
under N_2 unless stated otherwise. The tungsten catalyst was handled in a glove box. R_f values are obtained using thin layer chromatography (TLC) on silica gel-coated plates (Merck 60 F254) with the indicated eluens and compound were detected with UV-light, ammonium molybdate, potassium permanganate, ninhydrin, or anisaldehyde. Optical rotations were determined with a Perkin Elmer 241 polarimeter.

2,3,4,6-Tetra-*O*-benzyl-D-glucono-δ-lactone (23)



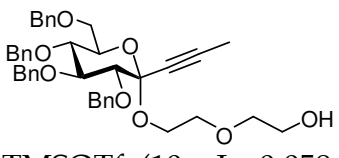
A solution of 2,3,4,6-tetra-*O*-benzyl-D-glucose (15.0 g, 27.7 mmol) in DMSO/Ac_2O (3/2 100 mL) was stirred at rt for 16 h and poured onto ice water. The water layer was extracted 3 times with an EtOAc/heptane solution (1/1). The organic layer was washed twice with water and once with brine. The organic layer was dried ($MgSO_4$) and concentrated. The crude product **23** (15 g, quant.) was used in the next step without further purification. R_f (1/2 EtOAc/heptane) = 0.49. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.39-7.16 (m, 20 H), 4.99 (d, *J* = 11.4 Hz, 1 H), 4.74-4.44 (m, 8 H), 4.12 (d, *J* = 6.5 Hz, 1 H), 3.97-3.89 (m, 2 H), 3.73 (dd, *J* = 2.4, 11.0 Hz, 1 H), 3.67 (dd, *J* = 3.3, 11.0 Hz, 1 H); in agreement with literature.[23]

1,2-Benzenedimethanol cyclic sulfate (28)



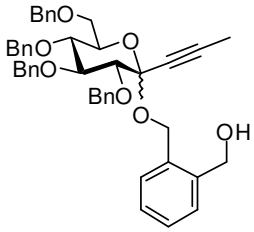
To a solution of the 1,2-benzenedimethanol (4.84 g, 35.0 mmol) and Et_3N (19.0 mL, 137 mmol) in CH_2Cl_2 (100 mL) at 0 °C was added a solution of $SOCl_2$ (3.85 mL, 52.7 mmol) in CH_2Cl_2 (10 mL), dropwise in 15 min. After 15 min the reaction was diluted with cold Et_2O (100 mL) and washed with cold water (2 × 50 mL), brine, dried ($MgSO_4$) and evaporated. Subsequently at 0 °C crude sulfite was dissolved in CCl_4/MeCN/H_2O (1/1/1.5 350 mL). $RuCl_3$ and $NaIO_4$ were added and the reaction was stirred for 1 h. The reaction was diluted with Et_2O (200 mL). The organic and water layer were separated and the water layer was extracted with Et_2O (100 mL). The organic layer was washed with brine, dried ($MgSO_4$) and evaporated. Recrystallization from heptane/acetone gave the desired cyclic sulfate **28** (4.90 g, 70%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ = 7.48-7.35 (m, 4 H), 5.44 (s, 4 H); in agreement with literature.[27, 31]

1-*O*-Diethyleneglycolyl-1-prop-1-ynyl-2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (25)



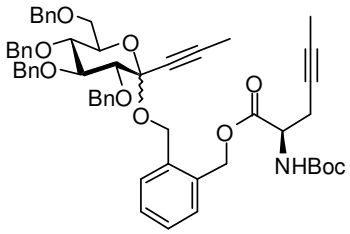
At 0 °C, a solution of compound **24** (0.15 g, 0.25 mmol) in CH_2Cl_2 (2 mL), was treated with $MgSO_4$ (0.13 g, 1.0 mmol), diethylene glycol (0.12 mL, 1.25 mmol), and TMSOTf (4.5 µL, 0.026 mmol). After stirring for 3 hours, additional TMSOTf (10 µL, 0.058 mmol) was added. The reaction was quenched with Et_3N (0.1 mL) and the solution was evaporated. Column chromatography afforded compound **25** (31 mg, 18%) and the dimer (41 mg). ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.38-7.25 (m, 20 H), 5.12-4.85 (m, 8 H), 4.77-3.41 (m, 14 H), 1.98 (s, 3 H).

1-*O*-(2-Methanolbenzyl)-1-prop-1-ynyl-2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (27)



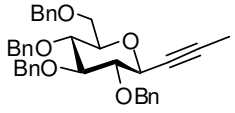
A solution of (*E,Z*)-1-bromo-1-propene (65 µL, 0.75 mmol) in dry THF (2.5 mL) was stirred at −78 °C. *n*-BuLi (0.69 mL, 1.1 mmol) was added and the reaction mixture was stirred for 2 h. A solution of lactone **23** (0.22 g, 0.40 mmol) in THF (2.5 mL) was added dropwise at −78 °C and the reaction mixture was stirred for 1 h. After the reaction was complete, cyclic sulfate **28** (0.15 g, 0.75 mmol) and HMPA (2.5 mL) were added. The reaction was stirred for 20 min at −78 °C and then slowly warmed to 0 °C. The reaction was stirred till completion and warmed to rt and was poured into aq. sat. NH_4Cl. The mixture was extracted with EtOAc (3 × 30 mL). The organic layer was evaporated and redissolved in THF (2.5 mL), before H_2SO_4 (50 µL) and H_2O (18 µL) were added. The reaction was stirred till completion and diluted with EtOAc. The organic layer was washed with saturated $NaHCO_3$, dried with $MgSO_4$ and evaporated. Purification by column chromatography afforded compound **27** (0.15 g, 0.21 mmol, 52%) as an α/β mixture (3:4). ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.38-7.25 (m,24 H), 5.12-4.85 (m, 8 H), 4.80-3.41 (m 10 H), 2.03 and 1.98 (2 s, 3 H).

1-*O*-(2-Methylhydroxyl[2-(*R*)-{*tert*-butoxycarbonylamino}hex-4-ynoate]benzyl) -1-prop-1-ynyl-2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (30)



A solution of Boc-2-butynylglycine (**29**, 73 mg, 0.32 mmol) in CH_2Cl_2 (2 mL) was stirred at 0 °C. DMAP (2.6 mg, 0.021 mmol), DCC (43 mg, 0.21 mmol) and compound **27** (0.15 g, 0.21 mmol) in CH_2Cl_2 (1 mL) were added and the reaction was stirred overnight at rt. The reaction mixture was filtered and the residue was washed with CH_2Cl_2. The organic layer was evaporated and the product was purified by column chromatography affording compound **30** (0.17 g, 0.19 mmol, 89%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.25-7.38 (m, 24 H), 5.46 (br d, 1 H), 5.12-4.85 (m, 9 H), 4.80-3.41 (m, 10 H), 2.75-2.81 (m, 2 H), 2.03 and 1.98 (2 s, 3 H), 1.68 (s, 3 H), 1.53 (s, 9 H).

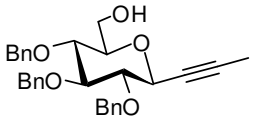
4,8-Anhydro-5,6,7,9-tetra-*O*-benzyl-1,2,3-trideoxy-D-glycero-L-mannonon-2-ynitol (31)



To a solution of (*E,Z*)-1-bromo-propene (0.90 mL, 10 mmol) in THF (35 mL) was added *n*-BuLi (0.60 M in hexane; 8.8 mL, 14 mmol) in 5 min at −78 °C. The solution was stirred for 1 h. A solution of lactone **23** (3.4 g, 6.4 mmol) in THF (35 mL) was added in 10 min. The reaction was stirred for ½ h at −78 °C, quenched with NH_4Cl (aq. sat.), warmed up to rt, extracted with EtOAc (2 × 100 mL), and washed with brine. Drying (Na_2SO_4) and concentrating, yielded the crude hemiacetal **24** (3.6 g, 6.2 mmol, 97%). Compound **24** was used for the next step without further purification. A solution of $BF_3{\cdot}OEt_2$ (3.8 mL, 30 mmol) and Et_3SiH (4.9 mL, 30 mmol) in MeCN/DCM (1/1, 30 mL) was added to a solution of the hemiacetal **24** in MeCN/DCM (1/1, 30 mL) in 15 min at 0 °C. After stirring at 0 °C for 1 h, the reaction was quenched with saturated $NaHCO_3$,

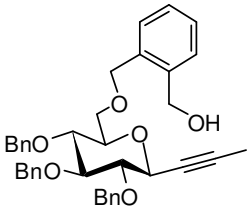
Extracted with EtOAc (2 × 150 mL), washed with brine, dried (Na_2SO_4) and concentrated and purified by column chromatography (1/5 EtOAc/heptane), to give **31** in 74% (2.6 g, 4.6 mmol) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.39-7.09 (m, 20 H), 5.00 (d, *J* = 10.5 Hz, 1 H), 4.91 (d, *J* = 11.0 Hz, 1 H), 4.83-4.79 (m, 3 H), 4.61 (d, *J* = 12.1 Hz, 1 H), 4.52 (dd, *J* = 11.3, 2.6 Hz, 2 H), 4.05-3.98 (m, 1 H), 3.77-3.52 (m, 5 H), 3.44-3.41 (m, 1 H), 1.88 (d, *J* = 2.1 Hz, 3 H); in agreement with literature.[30]

4,8-Anhydro-5,6,7-tri-*O*-benzyl-1,2,3-trideoxy-D-glycero-L-mannonon-2-ynitol (32)



To a solution of **31** (1.3 g 2.4 mmol) in Ac_2O (45 mL), H_2SO_4 (5% in Ac_2O, 10 mL) was added dropwise at −20 °C. The reaction was stirred for 10 min, quenched with aqueous NaOAc, diluted with EtOAc, and washed with saturated $NaHCO_3$, and brine. The organic phase was dried (Na_2SO_4), concentrated and purified by flash chromatography (1/5 EtOAc/heptane) to yield the acetolyzed product (1.0 g, 1.9 mmol) as a white solid. R_f (1/4 EtOAc/heptane) = 0.20. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.38-7.22 (m, 15 H), 5.01 (d, *J* = 10.6 Hz, 1 H), 4.95 (d, *J* = 10.9 Hz, 1 H), 4.87-4.80 (m, 3 H), 4.56 (d, *J* = 10.9 Hz, 1 H), 4.32 (d, *J* = 11.7 Hz, 1 H), 4.20 (dd, *J* = 4.4, 12.01 Hz, 1 H), 4.03 (dd, *J* = 2.1, 9.5 Hz, 1 H), 3.66-3.47 (m, 4 H), 2.04 (s, 3 H), 1.88 (d, *J* = 2.1 Hz, 3 H). ^{13}C-NMR (50 MHz, $CDCl_3$) δ = 171.0, 138.5, 138.1, 137.8, 128.7, 128.6, 128.4, 128.2, 128.1, 128.0, 128.0, 127.9, 86.1, 83.1, 82.7, 77.5, 77.0, 76.3, 75.9, 75.6, 75.3, 70.2, 63.6, 21.1, 4.0. $[\alpha]_D^{20}$ = +16.8 (c = 1.04, $CHCl_3$). IR (neat) ν = 3031, 2907, 2860, 2245, 1740. HRMS (CI) *m/z* calculated for $C_{32}H_{35}O_6$ $(M+H)^+$ 515.2434 found 515.2435. This was then dissolved in MeOH (40 mL) and KCN (15 mg, 0.23 mmol) was added. The reaction was stirred for 24 h. The mixture was neutralized with basic Amberlyst, filtrated and evaporated. The mixture was redissolved in EtOAc, washed with saturated NH_4Cl, dried ($MgSO_4$) and evaporated. Purification by flash chromatography (1/2 EtOAc/heptane) gave **32** as a white solid (0.75 g, 1.6 mmol, 66% from **31**. R_f (1/2 EtOAc/heptane) = 0.30. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.37-7.24 (m, 15 H), 4.99 (d, *J* = 10.6 Hz, 1 H), 4.92 (d, *J* = 11.1 Hz, 1 H), 4.87-4.80 (m, 3 H), 4.65 (d, *J* = 10.9 Hz, 1 H), 4.05 (dd, *J* = 1.6, 9.5 Hz, 1 H), 3.87 (dd, *J* = 2.3, 12.0 Hz, 1 H), 3.70-3.50 (m, 4 H), 3.35-3.31 (m, 1 H), 2.05 (br s, 1 H), 1.88 (d, *J* = 1.2 Hz, 3 H). ^{13}C-NMR (50 MHz, $CDCl_3$) δ = 138.6, 138.2, 138.0, 128.6, 128.5, 128.3, 128.1, 128.0, 128.0, 127.9, 127.8, 86.0, 83.0, 82.8, 79.4, 77.6, 76.5, 75.8, 75.6, 75.3, 70.1, 62.1, 3.9. $[\alpha]_D^{20}$ = –8.25 (c = 0.63, $CHCl_3$). IR (neat) ν = 3373, 3036, 2902, 2859, 2245. HRMS (CI) *m/z* calculated for $C_{30}H_{33}O_5$ $(M+H)^+$ 473.2328, found 473.2331.

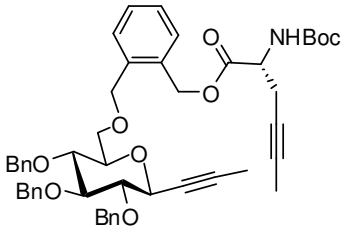
4,8-Anhydro-5,6,7-tri-*O*-benzyl-9-*O*-(2-methanolbenzyl)-1,2,3-trideoxy-D-glycero-L-mannonon-2-ynitol (33)



Compound **32** (0.76 g, 1.6 mmol) was dissolved in dry THF (50 mL) and cooled to −78 °C. KHMDS (0.5M in toluene, 4.8 mL, 2.4 mmol) was added and stirred for 30 min at −78 °C. Cyclic sulfate **28** (0.64 g, 3.2 mmol) was added. The temperature was raised overnight to rt. After stirring for 20 h, the reaction mixture

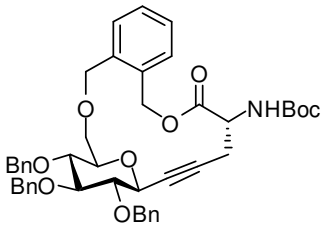
was poured into saturated NH_4Cl and extracted with EtOAc (2 × 100 mL). The sulfate was concentrated and redissolved in THF (100 mL). H_2SO_4 (0.40 mL) and H_2O (0.15 mL) were added and the reaction was stirred for 4 d. Half of the THF was evaporated, the reaction mixture was diluted with EtOAc (200 mL) and washed with water (3 × 100 mL), brine (200 mL), dried ($MgSO_4$) and evaporated. Purification by flash chromatography (1/2 EtOAc/heptane) afforded compound **33** (0.51 g, 54%) as a white solid. R_f (1/2 EtOAc/heptane) = 0.20. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.42-7.20 (m, 19 H), 4.98 (d, *J* = 10.6 Hz, 1 H), 4.90 (d, *J* = 11.1 Hz, 1 H), 4.84-4.76 (m, 3 H), 4.66 (d, *J* = 5.3 Hz, 2 H), 4.61 (d, *J* = 11.5 Hz, 2 H), 4.53 (d, *J* = 11.1 Hz, 1 H), 3.99 (dd, *J* = 2.0, 9.2 Hz, 1 H), 3.72 (dd, *J* = 2.0, 10.6 Hz, 1 H), 3.63-3.50 (m, 4 H), 3.43-3.40 (m, 1 H), 3.10 (br t, *J* = 6.1 Hz, 1 H), 1.88 (d, *J* = 2.2 Hz, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 140.6, 138.4, 138.0, 138.0, 135.7, 130.2, 129.8, 128.9, 128.4, 128.3, 128.2, 127.9, 127.8, 127.8, 127.7, 127.6, 86.1, 83.0, 82.7, 78.4, 77.8, 76.2, 75.8, 75.5, 75.2, 72.8, 70.3, 69.0, 63.7, 4.2. $[\alpha]_D^{20}$ = + 6.4 (c = 1.0, $CHCl_3$). IR (film) ν = 3480, 3062, 3027, 2905, 2868, 2250. HRMS (CI) *m/z* calculated for $C_{38}H_{41}O_6$ $(M+H)^+$ 593.2903, found 593.2875.

4,8-Anhydro-5,6,7-tri-*O*-benzyl-9-*O*-(2-methylhydroxyl[2-(*R*)-{*tert*-butoxycarbonylamino}hex-4-ynoate]benzyl)-1,2,3-trideoxy-D-glycero-L-mannonon-2-ynitol (34)



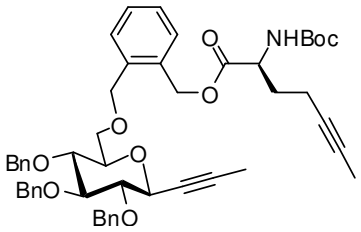
A solution of Boc-2-butynylglycine **29** (18 mg, 79 µmol) and alcohol **33** (34 mg, 56 µmol) in CH_2Cl_2 (1 mL) was stirred at 0 °C. DMAP (0.7 mg, 6 µmol) and DCC (15 mg, 73 µmol) in CH_2Cl_2 (1 mL) were added and the reaction was stirred overnight at rt. The reaction mixture was filtered, the residue was washed with CH_2Cl_2 and the filtrate was evaporated. Flash chromatography (1/4 EtOAc/heptane) afforded dialkyne **34** (42 mg, 94%) as a white solid. R_f (1/2 EtOAc/heptane) = 0.43. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.41-7.12 (m, 19 H), 5.34-5.25 (m, 3 H), 5.00 (d, *J* = 10.6 Hz, 1 H), 4.91 (d, *J* = 11.0 Hz, 1 H), 4.82-4.79 (m, 3 H), 4.64 (d, *J* = 12.4 Hz, 2 H), 4.50 (d, *J* = 10.8 Hz, 1 H), 4.45-4.42 (m, 1 H), 4.00 (dd, *J* = 1.7, 9.2 Hz, 1 H), 3.73 (d, *J* = 10.8 Hz, 1 H), 3.67 (dd, *J* = 4.5, 10.8 Hz, 1 H), 3.63-3.53 (m, 3 H), 3.43 (dd, *J* = 2.9, 9.2 Hz, 1 H), 2.72-2.56 (m, 2 H), 1.88 (d, *J* = 1.8 Hz, 3 H), 1.67 (t, *J* = 2.2 Hz, 3 H), 1.44 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.5, 154.9, 138.4, 138.1, 137.9, 136.4, 133.7, 129.3, 129.0, 128.3, 128.3, 128.1, 127.9, 127.8, 127.8, 127.7, 127.7, 127.6, 86.1, 82.7, 82.7, 80.1, 79.4, 78.9, 78.0, 76.675.8, 75.5, 75.2, 73.2, 71.1, 70.2, 69.3, 64.9, 52.6, 28.6, 23.4, 4.2, 3.9. $[\alpha]_D^{20}$ = +6.6 (c = 0.8, $CHCl_3$). IR (film) ν = 3723, 3425, 3058, 3028, 2967, 2911, 2855, 2250, 1740, 1722. HRMS (ESI) *m/z* calculated for $C_{49}H_{55}NNaO_9$ $(M+Na)^+$ 824.3775, found 824.3774.

RCAM product 35



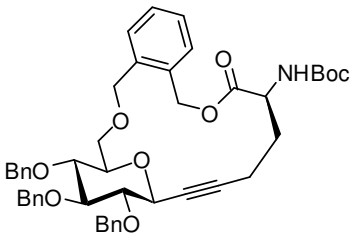
RCAM was performed under an argon atmosphere in a flame dried Schlenk flask. Dialkyne **34** (108 mg, 0.135 mmol) was co-evaporated with toluene (2 × 10 mL) and subsequently (^{t}BuO)$_3$W≡C^{t}Bu (**8**, 22 mg, 47 µmol) was added. This was dissolved in toluene (2 mL) and stirred for 30 minutes at 80 °C. The reaction was purified by flash chromatography (1/4 EtOAc/heptane) to yield cyclized product **35** (81 mg, 80%) as a solid. R_f (1/2 EtOAc/heptane) = 0.43. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.35-7.26 (m, 19 H), 5.34-5.23 (m, 3 H), 4.94-4.77 (m, 6 H), 4.71 (d, *J* = 11.7 Hz, 1 H), 4.62-4.58 (m, 2 H), 3.92 (d, *J* = 9.6 Hz, 2 H), 3.61-3.43 (m, 5 H), 2.96 (d, *J* = 16.4 Hz, 1 H), 2.56 (ddd, *J* = 2.1, 5.5, 16.6 Hz, 1 H), 1.39 (s, 9 H). ^{13}C-NMR (50 MHz, $CDCl_3$) δ = 171.1, 155.2, 138.5, 138.1, 138.0, 138.0, 137.5, 132.8, 130.2, 128.9, 128.6, 128.6, 128.5, 128.4, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 85.9, 82.2, 81.9, 81.4, 80.4, 79.5, 78.3, 75.9, 75.3, 75.3, 71.8, 69.7, 69.3, 65.2, 53.5, 28.4, 23.9. $[\alpha]_D^{20}$ = –30.0 (c = 0.26, $CHCl_3$). IR (film) ν = 3321, 3062, 3032, 2967, 2907, 2868, 2258, 1701. HRMS (ESI) *m/z* calculated for $C_{45}H_{49}NNaO_9$ $(M+Na)^+$ 770.3305, found 770.3366.

4,8-Anhydro-5,6,7-tri-*O*-benzyl-9-*O*-(2-methylhydroxyl[2-(*S*)-{*tert*-butoxycarbonylamino}hept-5-ynoate]benzyl)-1,2,3-trideoxy-D-glycero-L-mannonon-2-ynitol (38)



Coupling of **33** (16 mg, 27 µmol) with 3-pentynylglycine (7.9 mg, 32 µmol)[28] as described for **34** gave **38** (20 mg, 92%).R_f (1/2 EtOAc/heptane) = 0.43. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.41-7.12 (m, 19 H), 5.29 (d, *J* = 12.7 Hz, 1 H), 5.25 (d, *J* = 12.7 Hz, 1H), 5.13 (br d, *J* = 8.0 Hz, 1 H), 5.00 (d, *J* = 10.6 Hz, 1 H), 4.91 (d, *J* = 10.9 Hz, 1 H), 4.82-4.79 (m, 3 H), 4.68 (d, *J* = 12.3 Hz, 1 H), 4.59 (d, *J* = 12.3 Hz, 1 H), 4.50 (d, *J* = 10.9 Hz, 1 H), 4.42-4.36 (m, 1 H), 4.00 (dd, *J* = 2.1, 9.2 Hz, 1 H), 3.74-3.52 (m, 5 H), 3.44-3.41 (m, 1 H), 2.20-2.14 (m, 2 H), 2.06-1.98 (m, 1 H), 1.88 (d, *J* = 2.0 Hz, 3 H), 1.85-1.79 (m, 1 H), 1.74 (t, *J* = 2.4 Hz, 3 H), 1.42 (s, 9 H). ^{13}C-NMR (50 MHz, $CDCl_3$) δ = 172.2, 138.6, 138.3, 138.1, 136.5, 133.9, 129.4, 129.3, 128.6, 128.5, 128.5, 128.4, 128.2, 128.0, 128.0, 128.0, 127.9, 127.8, 86.1, 82.8, 82.7, 78.9, 77.9, 77.6, 76.9, 75.8, 75.5, 75.2, 71.1, 70.2, 69.3, 64.6, 53.1, 31.9, 28.4, 15.2, 3.9, 3.6. $[\alpha]_D^{20}$ = +5.6 (c = 1.25, $CHCl_3$). IR (film) ν = 3347, 3028, 2971, 2923, 2867, 2250, 1740, 1710. HRMS (ESI) *m/z* calculated for $C_{50}H_{57}NNaO_9$ $(M+Na)^+$ 838.3931, found 838.3914.

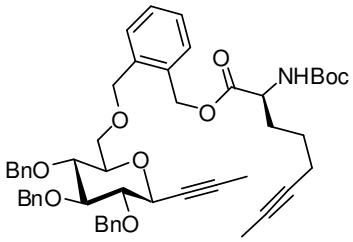
RCAM product 39



RCAM was performed on dialkyne **38** (20 mg, 25 µmol) as described for **35** to give product **39** (11 mg, 62%). R_f (1/2 EtOAc/heptane) = 0.43. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.37-7.27 (m, 19 H), 5.61 (d, *J* = 13.7 Hz, 1 H), 5.56 (d, *J* = 13.7 Hz, 1 H), 5.41 (br d, *J* = 6.2 Hz, 1 H), 4.99

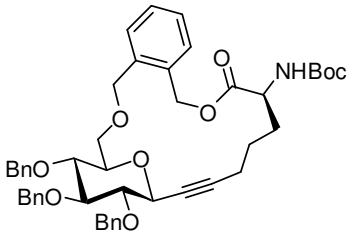
(d, *J* = 10.8 Hz, 1 H), 4.91 (d, *J* = 11.0 Hz, 1 H), 4.87-4.79 (m, 4 H), 4.73-4.71 (m, 1 H), 4.61 (d, *J* = 10.9 Hz, 1 H), 4.55-4.50 (m, 1 H), 3.92 (dt, *J* = 1.6, 9.5 Hz, 1 H), 3.75-3.57 (m, 5 H), 3.39 (dt, *J* = 3.1, 9.2 Hz, 1 H), 2.37-2.34 (m, 1 H), 2.22-2.18 (m, 1 H), 1.85-1.71 (m, 2 H), 1.42 (s, 9 H). ^{13}C-NMR (50MHz, $CDCl_3$) δ = 172.3, 155.2, 138.7, 138.4, 138.2, 135.9, 130.1, 128.8, 128.7, 128.5, 128.4, 128.3, 128.0, 128.0, 128.0, 127.9, 127.8, 86.2, 86.0, 84.5, 82.8, 82.7, 82.0, 79.0, 75.8, 75.5, 75.3, 73.4, 69.5, 68.4, 65.0, 52.1, 31.1, 28.5, 13.3. IR (film) ν = 3421, 3326, 3062, 3028, 2924, 2855, 2250, 1709. HRMS (ESI) *m/z* calculated for $C_{46}H_{51}NO_9Na$ $(M+Na)^+$ 784.3461, found 784.3440.

4,8-Anhydro-5,6,7-tri-*O*-benzyl-9-*O*-(2-methylhydroxyl[2-(S)-{*tert*-butoxycarbonylamino}oct-6-ynoate]benzyl)-1,2,3-trideoxy-D-glycero-L-mannonon-2-ynitol (40)



Coupling of **33** (2.6 mg, 4.3 µmol) with 4-hexynylglycine (1.3 mg, 5.2 µmol)[28] as described for the synthesis of **34** gave **40** (3.0 mg, 84%). R_f (1/2 EtOAc/heptane) = 0.43. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.40-7.11 (m, 19 H), 5.31 (d, *J* = 12.8 Hz, 1 H), 5.24 (d, *J* = 12.9 Hz, 1 H), 5.04-4.99 (m, 1 H), 4.99 (d, *J* = 10.6 Hz, 1 H), 4.91 (d, *J* = 10.9 Hz, 1 H), 4.82-4.79 (m, 3 H), 4.68 (d, *J* = 12.3 Hz, 1 H), 4.58 (d, *J* = 12.4 Hz, 1 H), 4.49 (d, *J* = 10.9 Hz, 1 H), 4.35-4.29 (m, 1 H), 4.00 (dd, *J* = 9.1, 2.1 Hz, 1 H), 3.72 (dd, *J* = 10.9, 1.8 Hz, 1 H), 3.68-3.40 (m, 5 H), 2.1-1.4 (m, 6 H), 1.88 (d, *J* = 2.1 Hz, 3 H), 1.74 (t, *J* = 2.5 Hz, 3 H), 1.42 (s, 9 H). IR (film) ν = 3295, 2919, 2863, 2254, 1750, 1718. HRMS (ESI) *m/z* calculated for $C_{51}H_{59}NO_9Na$ $(M+Na)^+$ 852.4088, found 852.4114.

RCAM product 41



RCAM was performed on dialkyne **40** (3.0 mg, 3.6 µmol) as described for **35** to give product **41** (0.8 mg, 29%). R_f (1/2 EtOAc/heptane) = 0.43. HRMS (ESI) *m/z* calculated for $C_{47}H_{53}NO_9Na$ $(M+Na)^+$ 798.3618, found 798.3606.

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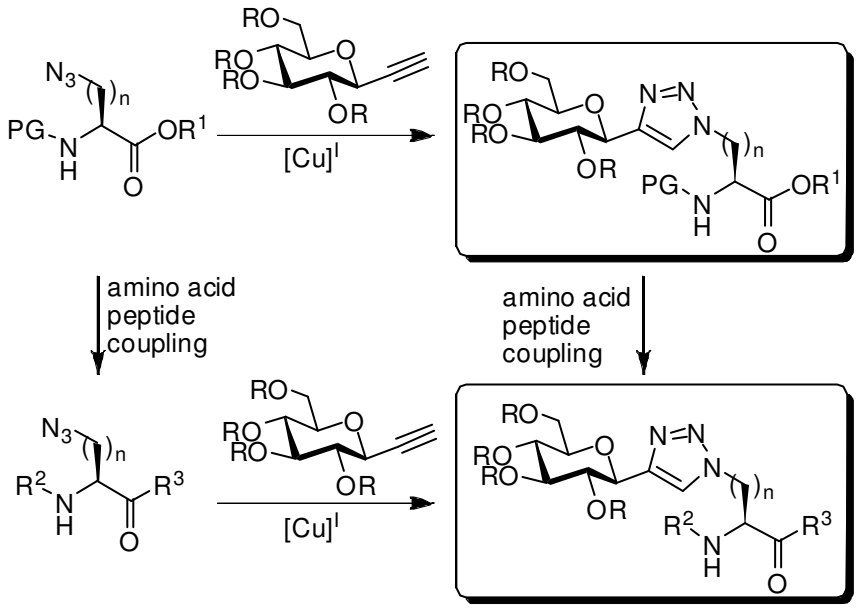
3 Expedient synthesis of triazole-linked glycoamino acids and peptides

Abstract

Methodology is presented for the synthesis of triazole-linked glycoamino acids and peptides based on copper(I)-catalyzed azide acetylene cycloaddition with glycoacetylenes and azidoamino acids. The resulting triazolyl glycoamino acids were suitable building blocks for the assembly of isosteric glycopeptides. An alternative route towards such triazolyl glycopeptides was also developed by late-stage introduction of the carbohydrate portion, involving similar cycloaddition of glycoacetylenes with azidopeptides.



Part of this chapter has been published: a) Kuijpers, B. H. M.; Groothuys, S.; Keereweer, A. R.; Quaedflieg, P. J. L. M.; Blaauw, R. H.; van Delft, F. L.; Rutjes, F. P. J. T. *Org. Lett.* **2004,** *6*, 3123-3126. b) Groothuys, S.; Kuijpers, B. H. M.; Quaedflieg, P. J. L. M.; Roelen, H. C. P. F.; Wiertz, R. W.; Blaauw, R. H.; van Delft, F. L.; Rutjes, F. P. J. T. *Synthesis* **2006**, 3146-3152. c) Kuijpers, B. H. M.; Groothuys, S.; Hawner, C.; ten Dam, J.; Quaedflieg, P. J. L. M.; Schoemaker, H. E.; van Delft, F. L.; Rutjes, F. P. J. T. *Org. Process Res. Dev.* **2008**, *12*, 503.

3.1 Introduction

In 2002 the groups of Meldal[1] and Sharpless[2] independently discovered that the 1,3-dipolar cycloaddition of an alkyne with an azide is effectively catalyzed by Cu(I)-salts. The original discovery, by Huisgen in 1967, involved heat-induced cycloaddition and usually led to a mixture of two regioisomers, *i.e.* the 1,4- and 1,5-regioisomer.[3] The copper(I)-catalyzed variation, in contrast, not only proceeds at ambient temperature, but also leads exclusively to the 1,4-substituted triazole. It is not surprising, therefore, that the procedure developed by Meldal and Sharpless, now postulated as the Cu(I)-mediated azide-alkyne cycloaddition (CuAAC), rapidly gained popularity in the synthetic community.

Among the many possibilities of application of triazoles in biologically relevant molecules[4,5] is the incorporation of a triazole as a bridging moiety in a glycoamino acid. Triazole-linked glycoamino acids may function as useful building blocks for the assembly of *N*-glycoproteins (**1**, Figure 3.1), in particular since the triazole function has been postulated as a useful amide isostere,[6,7] in terms of electronic properties and atom placement. Therefore, our research efforts were aimed at an efficient, high-yielding synthesis of triazole-linked isosteric glycopeptides such as **2** (Figure 3.1). The latter glycopeptide, termed ^{C}TGA from *C*-linked triazolylglycoamino acid, can be synthetically obtained by cycloaddition of acetylenic glycosides **3** and azidoamino acids **4**. In addition, besides the aim of merely constructing stable glycopeptide mimics, the resulting substituted triazole-linked glycopeptides more generally may display relevant biological activity against various targets.

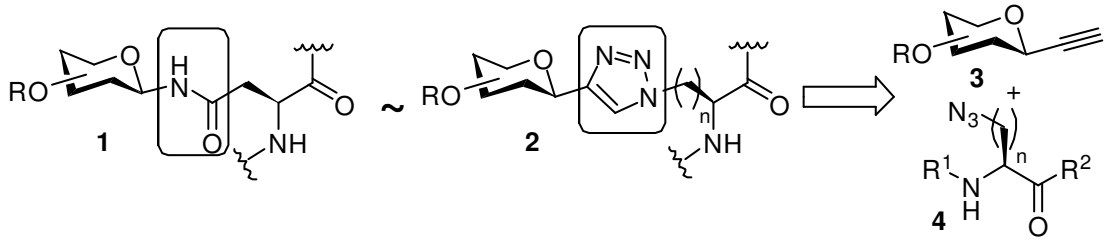
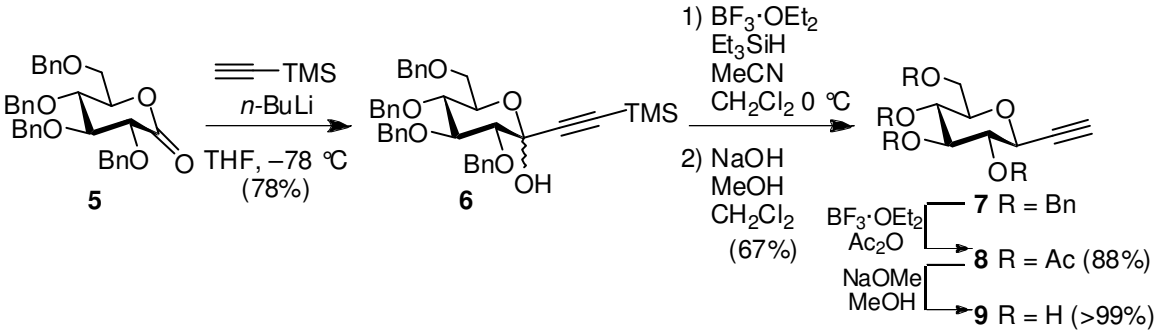


Figure 3.1 Retrosynthesis for isosteres of amide-linked glycoamino acids

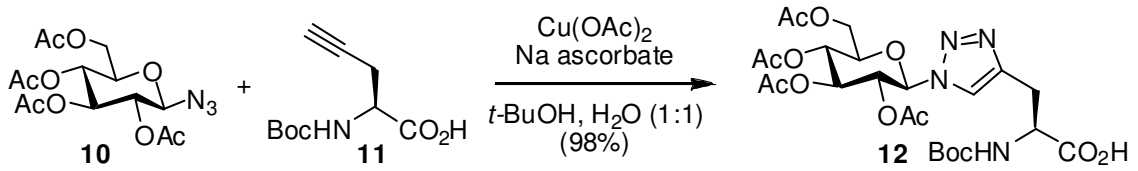
3.2 Triazole-linked *C*-glycoamino acids

From the retrosynthesis depicted in Figure 3.1 it becomes clear that the synthesis of triazolyl glycoproteins of type **2** requires the cycloaddition between a carbohydrate-derived acetylene and an azido-containing amino acid. Therefore, attention was first focused on the synthesis of glycoacetylenes of type **3**. To this end, the known lactone **5** (Section 2.6)[8] was converted into alkynylglucoside **6** (Scheme 3.1) by subjection to nucleophilic addition of Li-acetylide, generated *in situ* from TMS-acetylene and *n*-BuLi.[9] Hemi-acetal **6**, obtained as an α,β-mixture (ratio 1:1), was deoxygenated at the anomeric center under the action of $BF_3 \cdot OEt_2$ and Et_3SiH yielding stereoselectively the β-configured alkynyl glucoside **7**, after subsequent removal of the silyl group. Apart from the perbenzylated derivative **7**, be it a good building block for the CuAAC reaction, the acetylated and unprotected gluco-acetylenes **8** and **9** were also synthesized, bearing in mind the inevitable final deprotection steps to biologically relevant structures.



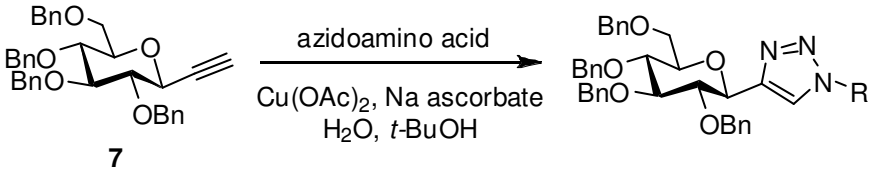
Scheme 3.1 Synthesis of β-glucoacetylenes **7-9**

The requisite azidoamino acids of type **4** were readily synthesized from the corresponding α-*N* protected L-lysine, L-ornithine, L-diamino-butanoic and propanoic acid via a diazotransfer reaction with TfN_3.[10]

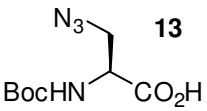
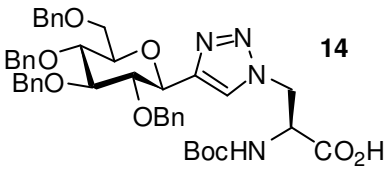
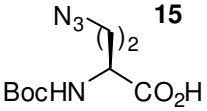
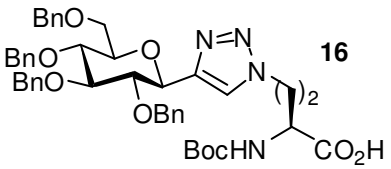
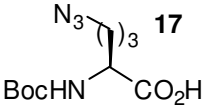
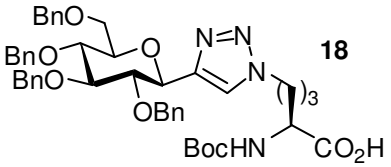
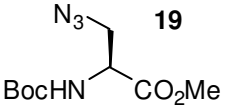
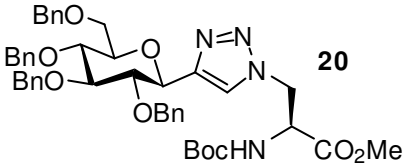
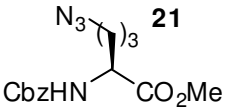
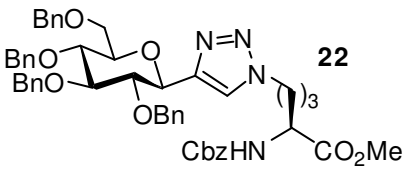
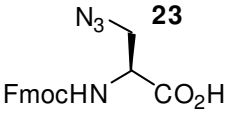
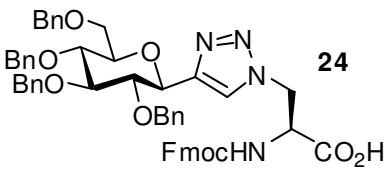
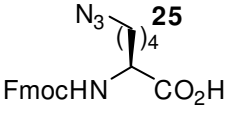
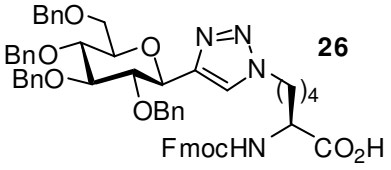


Scheme 3.2 Synthesis of triazole-linked *N*-glycoamino acid (^{N}TGA) **12**

Table 3.1 CuAAC reactions of glycoacetylene **7** and a variation of azidoamino acids



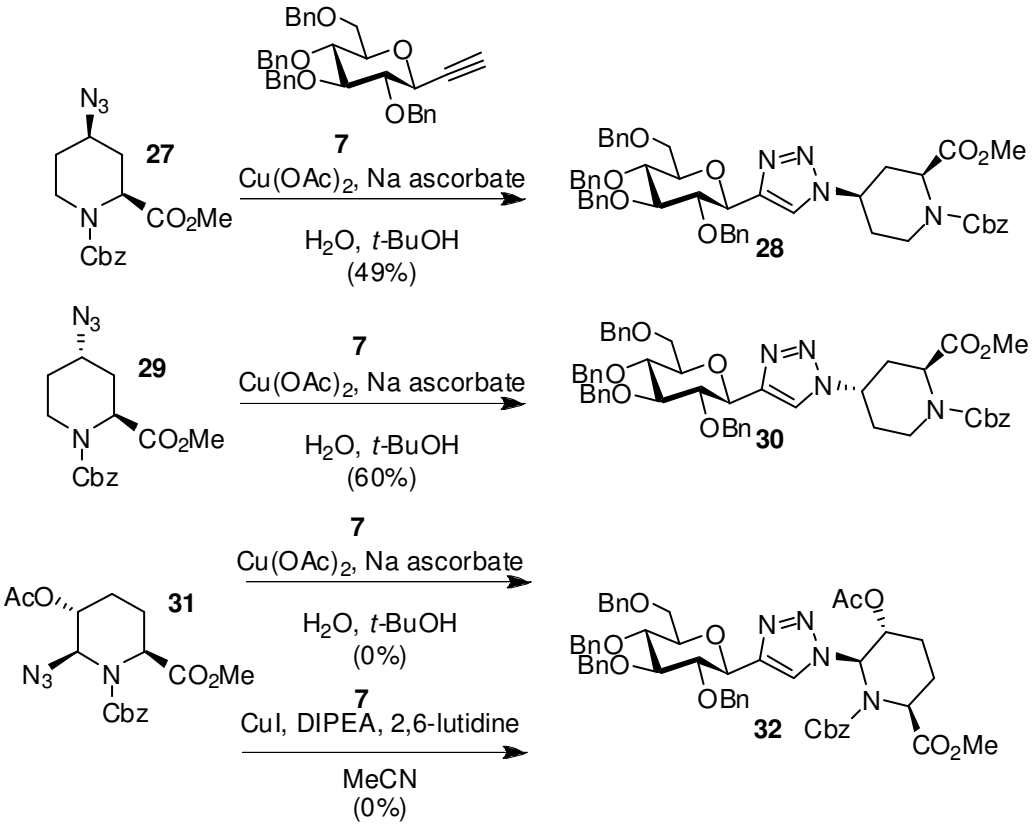
7

entry	azidoamino acid	[c]TGA	yield
1	 **13**	 **14**	70%
2	 **15**	 **16**	73%
3	 **17**	 **18**	71%
4	 **19**	 **20**	84%
5	 **21**	 **22**	87%
6	 **23**	 **24**	69%
7	 **25**	 **26**	71%

With the requisite building blocks in hand, several conditions were considered for the desired CuAAC. At an earlier occasion, it was found in our lab that optimal

CuAAC conditions for the inverse *N*-linked triazolylglycoamino acid, *i.e.* *N*-glycoamino acid (*N*TGA; Scheme 3.2) involved 0.2 equiv $Cu(OAc)_2$ and 0.4 equiv sodium ascorbate in a 1:1 (v/v) mixture of H_2O and *t*-BuOH (modified Sharpless conditions[2]).[11]

Applying the latter optimized conditions to glycoacetylene **7** and Boc-protected azidoamino acid **13**, provided the targeted Boc-T1M(4-GlcBn$_4$)-OH[12] **14** in 70% yield (entry 1, Table 3.1). Elongation of the amino acid side-chain had virtually no influence on the reactivity as can be inferred from Table 3.1, entries 2 and 3. Azidoamino acid derivatives containing a methyl ester appeared somewhat easier to purify than their free carboxylic acid counterparts, which may explain the slightly better yields (entries 4 and 5). Furthermore, it was shown that the reaction is compatible with a variety of the most commonly applied amine protective groups, *i.e.* Boc, Cbz and Fmoc.



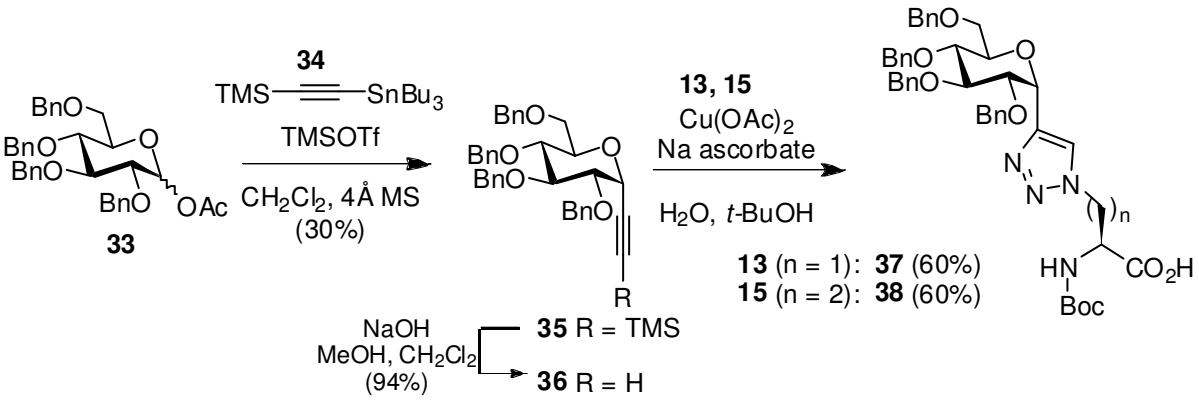
Scheme 3.3 Cycloaddition of pipecolic acids **27**, **29**, and **31** with glucoacetylene **7**

Apart from open-chain amino acids, cycloaddition of acetylene sugar **7** with the diastereomeric pipecolic acid derivatives **27** and **29** was also feasible, although with

lower efficiency (Scheme 3.3). The somewhat higher yield for the condensation with **29** with respect to **27** may be rationalized by the respective equatorial and axial orientation of the azide where the latter experiences more steric hindrance.

Unfortunately, 6-azidopipecolic acid derivative **31** failed to undergo cycloaddition with glucoacetylene **7**, neither under the influence of $Cu(OAc)_2$/Na ascorbate nor in the presence of CuI and base.[13] Presumably, steric hindrance from the neighboring Cbz protective group and 2,6-diaxial interaction with the methyl ester preclude access to the azide.

The above experiments firmly established the compatibility of the 1,3-dipolar cycloaddition with a range of protected amino acid derivatives, but led invariably to the β-linked triazole adduct. However, since *O*-linked glycoproteins are characterized by serine or threonine glycosylation with α-configuration, we became interested in expanding the scope of the sugar-amino acid CuAAC with α-configured acetylenes. Thus, synthesis of α-glucoacetylene **36** was accomplished by *C*-glycosidation (ethynylation) of sugar acetate **33** with 1-tributylstannyl-2-trimethylsilylacetylene **34**[14] in the presence of trimethylsilyl triflate followed by desilylation of the initial adduct as described by Dondoni *et al.* (Scheme 3.4).[15]

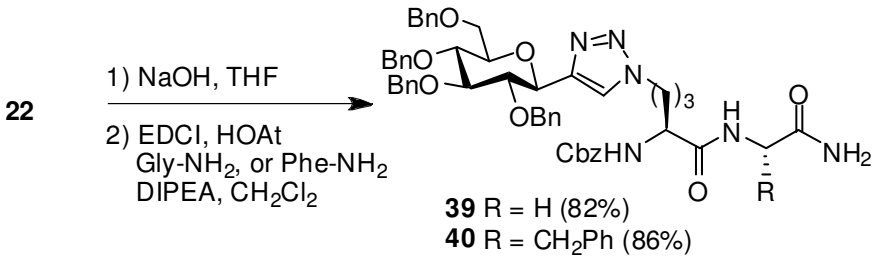


Scheme 3.4 Synthesis of α-glycoamino acids **37** and **38**

Much to our satisfaction, under the influence of the previously established conditions, α-glycoacetylene **36** was successfully coupled to azidoamino acid **13** and **15**, to obtain α-glycoamino acids **37** and **38**, respectively, in 60% yield (Scheme 3.4).

3.3 Triazole-linked glycopeptides

The smooth cycloaddition of acetylenic sugars with a range of amino acid derivatives encouraged us to corroborate whether CuAAC is also suitable for the synthesis of glycopeptides. In theory, two alternative strategies can be followed to achieve such a goal, involving either incorporation of a presynthesized glycoamino acid in a growing peptide chain, or 1,3-dipolar cycloaddition of glycoacetylenes onto azido-functionalized oligopeptides. Since several triazole-linked glycoamino acids were already at hand, the first strategy initially appeared most straightforward, as is illustrated in Scheme 3.5. The methyl ester of **22** was readily saponified to the carboxylic acid under the action of sodium hydroxide. Subsequent diimide-mediated coupling with glycine- and phenylalanine amide gave dipeptides **39** and **40** uneventfully, as an illustration that the triazolyl glycoamino acids behave normally under standard peptide coupling conditions.

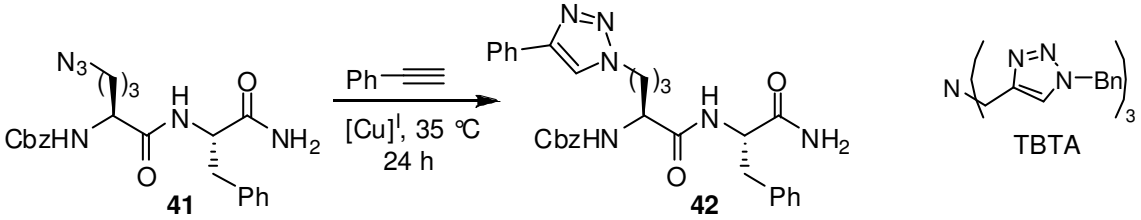


Scheme 3.5 Peptide coupling of Cbz-T1P(4-GlcBn$_4$)-OMe **22**

The alternative route, involving cycloaddition of dipeptides[16] and carbohydrate moieties, appeared less trivial beforehand, taking into consideration the potential incompatibility of *C*-terminal amide functionality with the Cu(I)-catalyzed 1,3-dipolar cycloaddition. To this end, a model dipeptide of azidopropylglycine and phenylalanine amide (**41**) was subjected to phenylacetylene under a range of conditions (Table 3.2). Indeed, the standard Cu(OAc)$_2$/Na ascorbate combination in a mixture of *t*-butyl alcohol and water, conditions that served our goal so well before, failed to lead to reaction in this case (entry 1). Since it was observed that both substrates were only poorly soluble in *t*-BuOH/H_2O, reaction was also performed in a THF/H_2O mixture, but also to no avail (entry 3). A likely explanation for the complete failure to undergo cycloaddition is complexation of copper(I), added in substoichiometric amount, to the free amide functionality. As a result of the

complexation, oxidation of Cu(I) to Cu(II) may occur, as may be inferred by the color change of the solution from yellow to blue. The latter observation led us to investigate the CuAAC in the presence of stoichiometric or excess CuI in acetonitrile or THF (entries 5-10). Unfortunately, also with an excess of a copper(I) species, in the presence of a variety of tertiary amines, resulted in only minor or no formation of the desired product (entries 5, 7 and 9). A more successful modification involved the addition of tris(benzyltriazolylmethyl)amine (TBTA), a ligand reported by Fokin *et al.*[17] to protect copper from oxidation and disproportionation, while enhancing its catalytic activity. Indeed, addition of TBTA under any of the previously applied conditions led to a dramatic improvement in yield as well as a significant decrease in reaction time. In particular, the use of a CuI/TBTA combination in the presence of Et_3N in acetonitrile resulted in a conversion of more than 90% of **41** into the desired Cbz-T1P(4-Ph)-Phe-NH_2 **42** (entry 8).

Table 3.2 Optimization of the 1,3-dipolar cycloaddition of dipeptide **41** with phenylacetylene

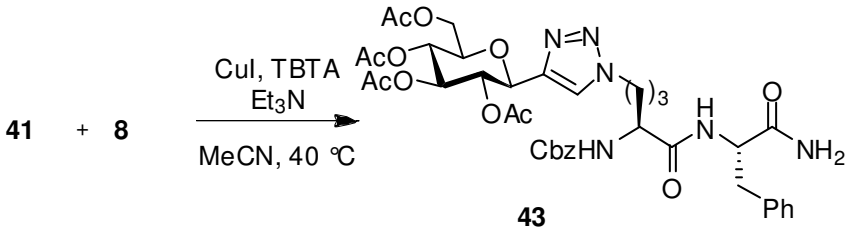


entry	Cu(I)-source	base	ligand	solvent	conv. [e]
1	$Cu(OAc)_2$, Na-asc[a]	-	-	*t*-BuOH, H_2O (1:1)	0%
2	$Cu(OAc)_2$, Na-asc[a]	-	TBTA[d]	*t*-BuOH, H_2O (1:1)	~50%
3	$Cu(OAc)_2$, Na-asc[a]	-	-	THF, H_2O (1:1)	0%
4	$Cu(OAc)_2$, Na-asc[a]	-	TBTA[d]	THF, H_2O (1:1)	~40%
5	CuI[b]	DIPEA, 2,6-lutidine[b]	-	MeCN	~30%
6	CuI[b]	DIPEA, 2,6-lutidine[b]	TBTA[b]	MeCN	~70%
7	CuI[b]	Et_3N[b]	-	MeCN	0%
8	CuI[b]	Et_3N[b]	TBTA[b]	MeCN	>90%
9	CuBr[c]	-	PMDETA[c]	THF	0%
10	CuBr[c]	-	TBTA[c]	THF	~70%

[a] 0.2 equiv. of $Cu(OAc)_2$ and 0.4 equiv. of Na-asc (Na ascorbate); [b] 2 equiv.; [c] 1 equiv.; [d] 0.2 equiv.;
[e] Conversion determined by TLC and LC-MS based on consumption of **41**

Having established suitable conditions for the cycloaddition of dipeptide **41** with phenylacetylene, condensation with glucose-derived acetylene **8** was investigated (Table 3.3). Although not applied to phenylacetylene, it was considered worthwhile to reduce the amount of copper iodide for the desired cycloaddition. Gratifyingly, it was found that reduction of CuI from two equivalents to one had no detectable effect on the conversion, reaching 100% conversion within 16 h (entry 1). With a further reduction to 50 mol% of copper, and even 20 mol%, complete conversion was still attained within 24 h (entry 2 and 3). Finally, only when the amount of copper was further reduced to 10 mol%, conversion failed to go to completion, even after 48 h (entry 4), whereas under the action of 1 mol% catalyst no conversion was observed at all (entry 5).

Table 3.3 Optimization of catalyst loading



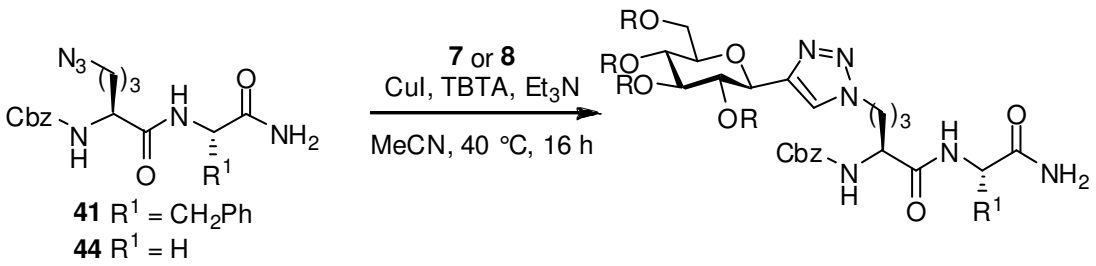
entry	loading[a]	concentration[b]	time	conversion[c]
1	100 mol%	1.1 mM	16 h	100%
2	50 mol%	1.8 mM	24 h	100%
3	20 mol%	12 mM	24 h	100%
4	10 mol%	3.7 mM	48 h	50%
5	1 mol%	5 mM	48 h	0%

[a] Loading of CuI, TBTA, and Et_3N; [b] Concentration of substrates
[c] Conversion determined by HPLC

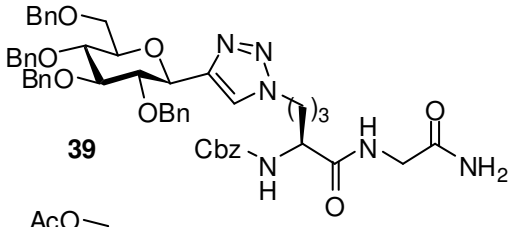
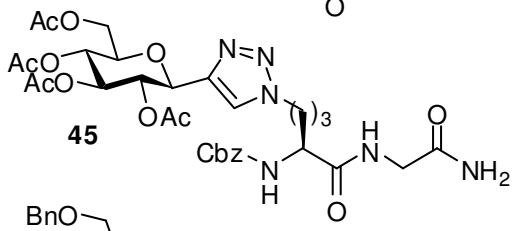
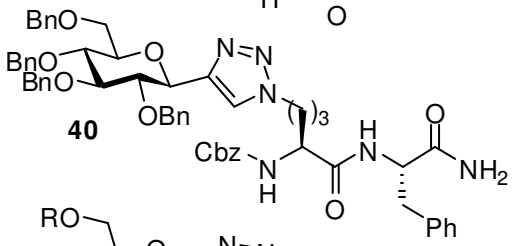
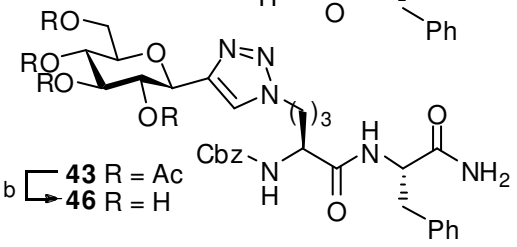
Since the reduction of the copper(I) catalyst loading below 20 mol% was found to be unsatisfactory for complete conversion to the product, subsequent couplings were performed under standardized conditions involving 20 mol% CuI, TBTA and Et_3N in acetonitrile. As summarized in Table 3.4, glycodipeptides **39**, **40**, **43** and **45** were all obtained in excellent yield upon cycloaddition of *N*-Cbz-protected azidodipeptides **41** and **44** with acetylenic *C*-glycosides **7** and **8**, irrespective of the carbohydrate *O*-protective groups (acetyl in **8** and benzyl in **7**). Deprotection of the acetyl

protective groups of **43** using K_2CO_3/MeOH, proceeded smoothly to give the unprotected triazole-linked glycodipeptide **46** (Table 3.4).

Table 3.4 CuAAC reaction of dipeptides **41** and **44** with glycoacetylenes **7** and **8** and deprotection of **43**



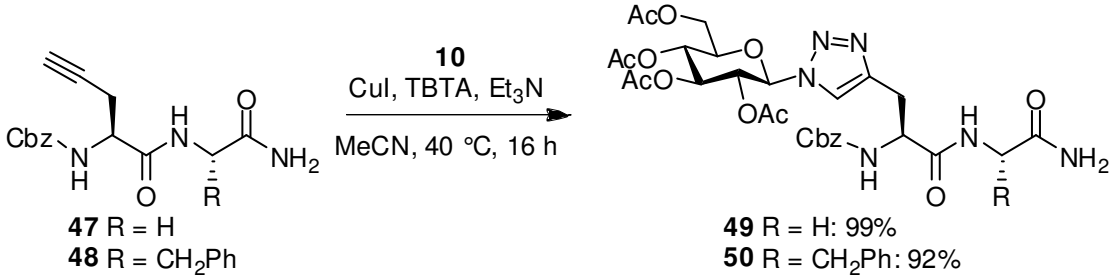
41 $R^1 = CH_2Ph$
44 $R^1 = H$

entry	carbohydrate	R^1	glycodipeptide	yield
1	**7**	H (**44**)	 **39**	96%
2	**8**		 **45**	62%[a]
3	**7**	CH_2Ph (**41**)	 **40**	87%
4	**8**		 b **43** R = Ac, **46** R = H	88%

20 mol% CuI, TBTA, and Et_3N; [a] Conversion ca. 70%; [b] K_2CO_3, MeOH, 95%

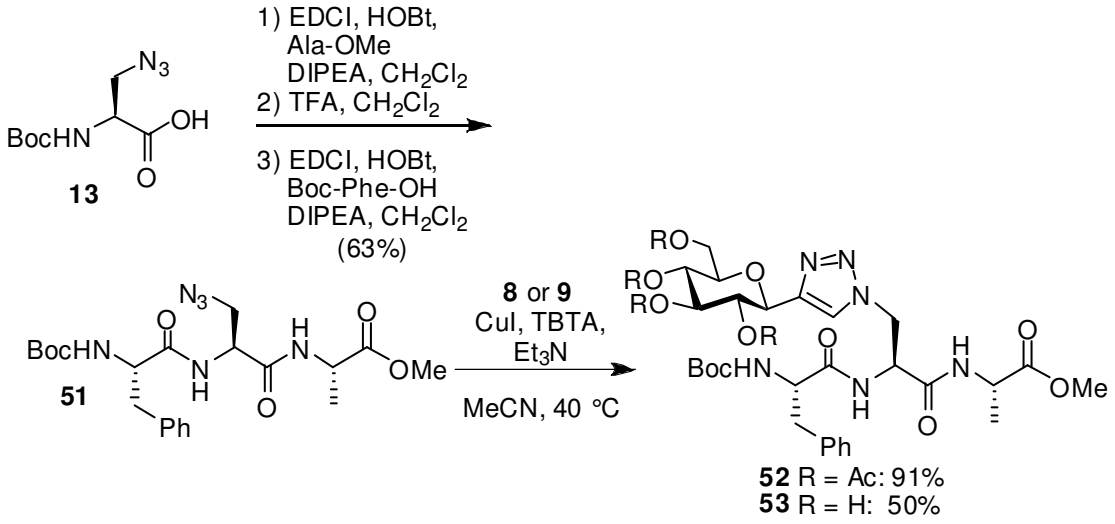
Whereas the examples so-far all involve cycloaddition of an azidopeptide with an acetylenic sugar, the alternative combination, *i.e.* CuAAC of an azidosugar with an acetylenic dipeptides was also investigated. Not unexpectedly, the desired

glycodipeptides **49** and **50** (Scheme 3.6) were formed uneventfully and in excellent yield upon subjecting acetylenic dipeptides **47** and **48**, respectively, to **10**.



Scheme 3.6 CuAAC reaction of dipeptides **47** and **48** with glucosyl azide **10**

A final example of the versatility of CuAAC for the synthesis of triazolyl glycopeptides is provided in the synthesis of a tripeptide with a central azidoalanine (Scheme 3.7). Cycloaddition to glycoacetylenes **8** and **9** resulted in the desired glycopeptides **52** and **53**. In case of the fully unprotected glycoacetylene **9**, the moderate yield could possibly be due to the fact that the highly hydrophilic character of **53** thwarted effective extraction during work-up (Scheme 3.7).



Scheme 3.7 CuAAC reaction of tripeptide **51** with glycoacetylenes **8** and **9**

3.4 Conclusions

Triazole-linked glycoamino acids of either α- or β-configuration were successfully prepared by application of Cu(I)-catalyzed 1,3-dipolar cycloaddition of alkynylsugars with a variety of azidoamino acids. The methodology was extended to

the synthesis of glycopeptides in two alternative strategies, involving either incorporation of triazole-linked glucoamino acids in a peptide chain or peptide coupling prior to cycloaddition with gluco-acetylenes. Both strategies successfully led to the desired glycopeptides, with no substantial difference in overall yield. In case of cycloaddition to *C*-terminal dipeptide amides, TBTA was found to be an essential ligand to stabilize the Cu(I) species and catalyze the cycloaddition, thus providing excellent conditions for the high-yielding assembly of triazolylglycopeptides.

3.5 Acknowledgements

Dr. Brian Kuijpers is kindly acknowledged for his contribution to the synthesis of ^{N}TGAs and for fruitful discussions about the 1,3-dipolar cycloadditions. Jeroen ten Dam is kindly acknowledged for the coupling with the pipecolic acids. Marta Juan y Seva is kindly acknowledged for the synthesis of the triazole-linked glycotripeptides. We are grateful to Dr. Richard Blaauw (Chiralix BV) for providing the pipecolic acids **27**, **29** and **31**. DSM (Geleen, The Netherlands) is kindly acknowledged for the DSC measurements of azido-containing compounds.

3.6 Experimental section

For general experimental details, see Section 2.6. Thermal analysis of representative azide-containing compounds has shown that upon warming exothermic decomposition may take place at onset temperatures lying between 70 and 150 °C. As an example, DSC measurements showed that compound **15** has an onset temperature of 70 °C, at which a strongly exothermic (576.6 kJ/kg) reaction takes place. Therefore, special caution has to be taken when handling these compounds.

Nomenclature of the triazole-linked glycoamino acids.
For the sake of clarity the names of the peptides were abbreviated, applying the standard three letter codes for natural amino acids. For the non-natural amino acids we derived a code as depicted in Figure 3.2. For example, **T4M** stands for a **t**riazol-**4**-yl-**m**ethylglycine containing amino acid and **t**riazol-**1**-yl-**b**utylglycine was abbreviated as **T1B**. Any substituents are presented in brackets.

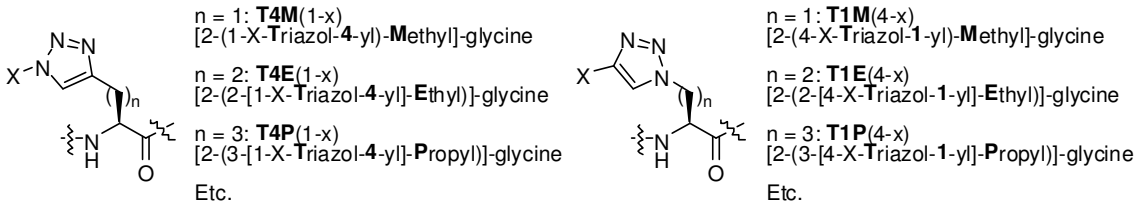


Figure 3.2 Nomenclature of the triazole-linked glycoamino acids.

General procedure A (cycloaddition)

To a solution of the glycosyl acetylene (1 equiv) and the amino acid derivative (1 equiv) in *t*-BuOH (0.5 M) was added a mixture of $Cu(OAc)_2$ (20 mol%) and sodium ascorbate (40 mol%) in H_2O (0.04 M and 0.08 M, respectively). The reaction was stirred overnight, water was added and the product was extracted with EtOAc (2 ×). The combined organic layers were washed with water, dried (Na_2SO_4) and concentrated. The product was purified by flash chromatography using EtOAc/heptane mixtures.

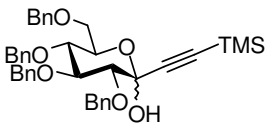
General procedure B (optimized cycloaddition)

To a solution of the peptide (25 µmol) and the carbohydrate (25 µmol) in MeCN (0.5 mL) were added solutions of Et_3N in MeCN (10 mM, 0.50 mL, 0.20 equiv) and TBTA in MeCN (10 mM, 0.50 mL, 0.20 equiv). Nitrogen was bubbled through the solution for 5 min. A solution of CuI in MeCN (10 mM, 0.50 mL, 0.20 equiv) was added and the reaction mixture was stirred at 40 °C for 16 h. Brine (2 mL) was added and the mixture was extracted with EtOAc (2 × 4 mL). The combined organic layers were dried ($MgSO_4$) and concentrated. The product was purified by gradient flash chromatography ($MeOH/CH_2Cl_2$).

General procedure C (peptide coupling)

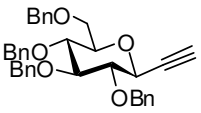
Cbz-amino acid (1 mmol) was dissolved in DMF (2.5 mL). To the clear cooled (0-5 °C) solution was added DIPEA (0.35 mL, 2.1 mmol) followed by EDCI (211 mg, 1.1 mmol) and HOAt (150 mg, 1.1 mmol) and the mixture was stirred for at least 0.5 h at 0-5°C. Subsequently, a solution of Phe-NH_2 or Gly-NH_2 (1.1 mmol) in DMF (2.5 mL) was added and the mixture was stirred for 1 h at 0-5 °C. The mixture was warmed to ambient temperature and stirred overnight. TLC analysis (EtOAc) showed complete conversion of the carboxylic acid starting material. The reaction mixture was partitioned between EtOAc (75 mL) and H_2O (15 mL) to which a few drops of 1 N aqueous HCl were added. The aqueous layer was extracted with EtOAc (40 mL) and the combined organic phase was washed with 0.5 N aqueous HCl (40 mL), 1 M aqueous $KHCO_3$ (40 mL) and brine (40 mL) and subsequently dried (Na_2SO_4) and concentrated.

1-[(Trimethylsilyl)ethynyl]-2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (6)



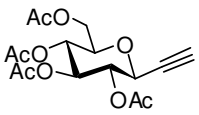
TMS-acetylene (9.0 mL, 61 mmol) was dissolved in THF (250 mL) and cooled to −78 °C. *n*-BuLi (44 mL, 71 mmol, 1.6 M in hexane) was added in 15 min and the reaction mixture was stirred for 45 min. The tetra-*O*-benzylglucolactone **5** (25.5 g, 47.3 mmol) was dissolved in THF (250 mL) and added dropwise to the reaction mixture in 15 min. The reaction was stirred for 30 min, and quenched with saturated NH_4Cl at −78 °C, extracted with EtOAc, washed with water and brine, dried ($MgSO_4$) and concentrated. The product was purified by flash chromatography with EtOAc/heptane (1/2) and afforded the hemiacetal **6** (26.5 g, 41.5 mmol, 88%) as a colorless oil. R_f (EtOAc/heptane 1/2) = 0.49. ^{1}H-NMR ($CDCl_3$, 400 MHz, α/β = 1/1.44) δ: 7.38-7.12 (m, 20 H), 5.07-5.03 (m, 1 H), 4.93-4.75 (m, 4 H), 4-64-4.60 (m, 1 H), 4.56-4.48 (m, 2 H), 4.03-3.84 (m, 2 H), 3.77-3.63 (m, 4 H), 3.51-3.48 (m, 1 H), 0.21 and 0.18 (s, 9H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 138.7, 138.5, 138.0, 137.9, 137.8, 137.7, 128.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.6, 127.5, 127.5, 127.4, 127.4, 127.2, 104.0, 100.7, 95.4, 93.5, 91.3, 89.0, 84.2, 84.1, 83.5, 82.4, 77.7, 77.4, 75.9, 75.7, 75.6, 75.1, 75.0, 74.3, 74.1, 73.4, 73.4, 72.0, 68.6, 68.5, 0.2, -0.0. Both are in agreement with literature.[9]

1-Ethynyl-1-deoxy-2,3,4,6-tetra-*O*-benzyl-*β*-D-glucopyranose (7)



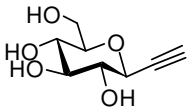
At 0 °C, a solution of $BF_3{\cdot}OEt_2$ (13.5 mL, 108 mmol) and Et_3SiH (17 mL, 107 mmol) in $MeCN/CH_2Cl_2$ (1/1, 200 mL) was added in 15 min to a solution of hemiacetal **6** (13.7g, 21.5 mmol) in $MeCN/CH_2Cl_2$ (1/1, 200 mL). The reaction was stirred for 30 min and quenched with saturated $NaHCO_3$, extracted with EtOAc, washed with brine, dried ($MgSO_4$) and concentrated. Purification by flash chromatography with EtOAc/heptane (1/5) afforded glucosyl TMS acetylene (11.5 g, 86%). R_f (EtOAc/heptane 1/5) = 0.42. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.38-7.11 (m, 20 H), 5.04 (d, *J* = 10.4 Hz, 1 H), 4.91 (d, *J* = 11.0 Hz, 1 H), 4.83-4.77 (m, 3 H), 4.62 (d, *J* = 12.2 Hz, 1 H), 4.55-4.50 (m, 2 H), 4.08-4.05 (m, 1 H), 3.74 (dd, *J* = 10.8, 1.9 Hz, 1 H), 3.68 (dd, *J* = 10.8, 4.3 Hz, 1 H), 3.66-3.55 (m, 3 H), 3.45-3.38 (m, 1 H), 0.18 (s, 9 H). Glucosyl TMS acetylene (11.5 g, 17.7 mmol) was dissolved in $MeOH/CH_2Cl_2$ (5/1, 120 mL), and a 1 M NaOH solution (35 mL) was added. The reaction was stirred for 1 h, quenched with 1 M HCl (35 mL), diluted with EtOAc (150 mL), washed with water, dried ($MgSO_4$) and concentrated. Purification by gradient flash chromatography (EtOAc, heptane) afforded glucoacetylene **7** (7.5 g, 77%) as a white solid. R_f (1/5 EtOAc/heptane) = 0.24. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.38-7.10 (m, 20 H), 5.00 (d, *J* = 10.4 Hz, 1 H), 4.91 (d, *J* = 11.0 Hz, 1 H), 4.85-4.78 (m, 3 H), 4.61 (d, *J* = 12.1 Hz, 1 H), 4.53 (d, *J* = 11.8 Hz, 2 H), 4.06-4.02 (m, 1 H), 3.74 (dd, *J* = 10.9, 2.1 Hz, 1 H), 3.69 (dd, *J* = 10.9, 4.3 Hz, 1 H), 3.66-3.57 (m, 3 H), 3.44 (ddd, *J* = 7.5, 4.1, 2.0 Hz, 1 H), 2.53 (d, *J* = 2.2 Hz, 1 H). In agreement with literature.[18]

1-Ethynyl-1-deoxy-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (8)



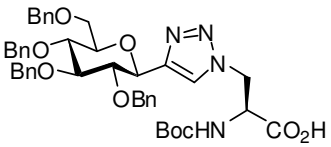
To a solution of tetra-*O*-benzyl glycoacetylene **7** (2.80 g, 5.11 mmol) in Ac_2O (80 mL) was added $BF_3 \cdot OEt_2$ (6.15 mL, 48.8 mmol). The reaction was stirred for 2.5 h, cooled to 0 °C quenched with saturated $NaHCO_3$ and extracted with EtOAc (3 × 150 mL). The organic layer wash washed with brine, dried ($MgSO_4$) and concentrated. Purification by flash chromatography with EtOAc/heptane (1/2) gave tetra-*O*-acetyl glycoacetylene **8**. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 5.22-5.07 (m, 3 H), 4.24 (dd, *J* = 12.5, 4.8 Hz, 1 H), 4.22-4.19 (m, 1 H), 4.13 (dd, *J* = 12.5, 2.2 Hz, 1 H), 3.68 (ddd, *J* = 9.8, 4.8, 2.2 Hz, 1 H), 2.52 (d, *J* = 2.2 Hz, 1 H), 2.10 (s, 1 H), 2.07 (s, 1 H), 2.03 (s, 1 H), 2.01 (s, 1 H). In agreement with literature.[19]

1-Ethynyl-1-deoxy-β-D-glucopyranose (9)



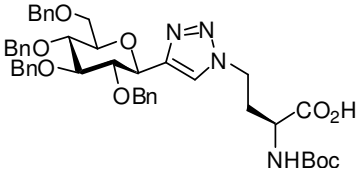
A solution of **8** (0.815 g, 2.29 mmol) in MeOH (23 mL) was treated with a NaOMe (13.2 mL, 5.8 M in MeOH) at rt over night. The crude mixture was neutralized with Amberlite IR-120 (H^+) (prewashed with MeOH), filtered and evaporated to obtain the desired product **9** (0.457 g, >99%). R_f(MeOH/CH_2Cl_2 1/9) = 0.12. ^{1}H-NMR (400 MHz, CD_3OD) δ = 3.95-3.92 (m, 1 H), 3.86 (dd, *J* = 1.5 12.0 Hz, 1 H), 3.65 (dd, *J* = 5.2, 12.0 Hz, 1 H), 3.33-3.23 (m, 4 H), 2.89 (d, *J* = 2.1 Hz, 1 H). In agreement with literature.[19]

Boc-L-T1M{4-[β-D-Glc(Bn)$_4$]}-OH (14)



Applying general procedure A gave **14** as a white solid (54 mg, 0.07 mmol, 70%). R_f (1% AcOH in EtOAc) = 0.46. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.52 (s, 1 H), 7.30-6.97 (m, 20 H), 5.41 (br d, *J* = 5.3 Hz, 1H), 4.92-4.73 (m, 5 H), 4.57-4.46 (m, 6 H), 4.17 (d, *J* = 10.8 Hz, 1 H), 3.90 (t, *J* = 9.1 Hz, 1 H), 3.79 (t, *J* = 8.8 Hz, 1 H), 3.72-3.57 (m, 4 H), 1.41 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.3, 155.2, 145.0, 138.3, 137.9, 137.7, 137.6, 129.0, 128.3, 128.3, 128.2, 128.0, 127.9, 127.9, 127.8, 127.7, 127.7, 127.6, 127.5, 125.2, 124.6, 86.8, 81.8, 80.8, 79.4, 78.1, 75.8, 75.2, 75.0, 73.8, 73.6, 69.1, 54.0, 51.4, 28.6. IR (film) ν = 3399, 3058, 2885, 2362, 2327, 1735 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{44}H_{51}N_4O_9$ $(M+H)^+$ 779.3656, found 779.3648. $[\alpha]_D$ = +35.5 (c = 0.68, $CHCl_3$).

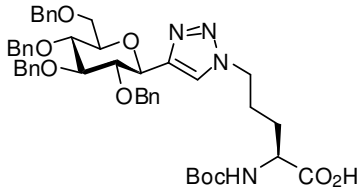
Boc-L-T1E{4-[β-D-Glc(Bn)$_4$]}-OH (16)



Applying general procedure A gave **16** as a white solid (58 mg, 0.07 mmol, 73%). R_f (1% AcOH in EtOAc) = 0.53. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 8.93 (br s, 1 H), 7.60 (s, 1 H), 7.31-6.96 (m, 20 H), 5.55 (br s, 1 H), 4.92 (d_{AB}, *J* = 11.3 Hz, 1 H), 4.88 (d_{AB}, *J* = 11.5 Hz, 1 H), 4.84 (d_{AB}, *J* = 10.7 Hz, 1 H), 4.62 (d_{AB}, *J* = 10.7 Hz, 1 H), 4.55-4.24 (m, 8 H), 3.90 (dd, *J* = 9.0, 8.6 Hz, 1 H), 3.79 (dd, *J* = 8.6, 8.6 Hz, 1 H), 3.71-3.60 (m, 4 H), 2.47 (br s, 1 H), 2.19 (br s, 1 H), 1.45 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 173.2, 155.7, 145.1, 138.4, 137.9, 137.8, 137.7, 128.3, 128.1, 127.9, 127.7, 127.6, 124.0, 87.0, 81.8, 80.5, 79.4, 78.2, 75.7, 75.2, 74.9, 73.8, 73.4, 69.1, 51.4, 47.1, 33.8, 28.6. IR (film) ν = 3391, 3019, 2902, 2859, 2353, 2327,

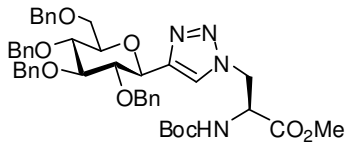
1714 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{45}H_{53}N_4O_9$ $(M+H)^+$ 793.3812, found 793.3854. $[\alpha]_D$ = +21.0 (c = 1.06, $CHCl_3$).

Boc-L-T1P{4-[β-D-Glc(Bn)$_4$]}-OH (18)



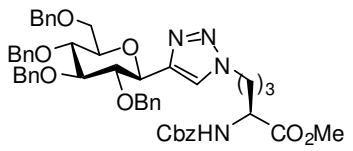
Applying general procedure A gave **18** as a white solid (59 mg, 0.07 mmol, 71%). R_f (1% AcOH in EtOAc) = 0.59. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 9.87 (br s, 1 H), 7.42 (s, 1 H), 7.29-6.98 (m, 20 H), 5.26 (d, *J* = 7.64 Hz, 1 H), 4.93 (d_{AB}, *J* = 11.1 Hz, 1 H), 4.89 (d_{AB}, *J* = 11.1 Hz, 1 H), 4.84 (d_{AB}, *J* = 10.9 Hz, 1 H), 4.62 (d_{AB}, *J* = 10.9 Hz, 1 H), 4.57-4.52 (m, 3 H), 4.48 (d_{AB}, *J* = 12.3 Hz, 1 H), 4.28 (d_{AB}, *J* = 10.9 Hz, 1 H), 4.30-4.10 (m, 3 H), 3.89 (dd, *J* = 9.2, 9.2 Hz, 1 H), 3.81 (dd, *J* = 9.0, 8.8 Hz, 1 H), 3.74-3.67 (m, 3 H), 3.63-3.59 (m, 1 H), 1.92-1.62 (m, 4 H), 1.42 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 173.7, 155.0, 145.0, 138.0, 137.4, 137.3, 137.3, 128.5, 127.9, 127.9, 127.8, 127.7, 127.7, 127.5, 127.4, 127.4, 127.2, 127.2, 127.1, 127.1, 124.8, 122.5, 86.5, 81.3, 79.8, 78.9, 77.8, 75.3, 74.7, 74.4, 73.5, 73.0, 68.7, 52.3, 49.4, 29.3, 28.2, 25.9. IR (film) ν = 3417, 3334, 3062, 3028, 2920, 2863, 2362, 2332, 2237, 1701 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{46}H_{54}N_4NaO_9$ $(M+Na)^+$ 829.3788, found 829.3787. $[\alpha]_D$ = +11.2 (c = 0.92, $CHCl_3$).

Boc-L-T1M{4-[β-D-Glc(Bn)$_4$]}-OMe (20)



Applying general procedure A gave **20** as a white solid (175 mg, 0.22 mmol, 84%). R_f (1/1 EtOAc/heptane) = 0.40. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.43 (s, 1 H), 7.33-7.00(m, 20 H), 5.31 (br d, *J* = 6.2 Hz, 1 H), 4.94 (d_{AB}, *J* = 11.1 Hz, 1 H), 4.90 (d_{AB}, *J* = 11.1 Hz, 1 H), 4.85 (d_{AB}, *J* = 10.8 Hz, 1 H), 4.86-4.80 (m, 1 H), 4.75-4.65 (m, 2 H), 4.61 (d_{AB}, *J* = 11.7 Hz, 1 H), 4.58 (d_{AB}, *J* = 10.9 Hz, 1 H), 4.56 (d_{AB}, *J* = 12.1 Hz, 1 H), 4.51 (d, *J* = 9.7 Hz, 1 H), 4.51 (d_{AB}, *J* = 12.1 Hz, 1 H), 4.28 (d_{AB}, *J* = 10.7 Hz, 1 H), 3.92 (dd, *J* = 9.4, 9.2 Hz, 1 H), 3.81 (dd, *J* = 9.0, 8.8 Hz, 1 H), 3.75-3.67 (m, 6 H), 3.63-3.60 (m, 1 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 181.1, 168.7, 154.4, 145.2, 138.0, 137.4, 137.4, 127.9, 127.8, 127.7, 127.4, 127.3, 127.2, 127.2, 127.1, 123.6, 86.5, 81.4, 80.4, 79.1, 77.8, 75.3, 74.7, 74.5, 73.6, 73.1, 68.8, 53.5, 52.8, 50.6, 28.1. IR (film) ν = 3028, 2924, 2850, 2353, 2332, 1748, 1714 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{45}H_{52}N_4NaO_9$ $(M+Na)^+$ 815.3632, found 815.3615. $[\alpha]_D$ = +21.9 (c = 0.96, $CHCl_3$).

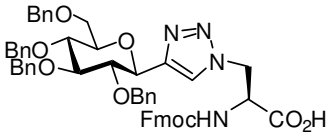
Cbz-L-T1P{4-[β-D-Glc(Bn)$_4$]}-OMe (22)



Applying general procedure A gave **22** (10.2 g, 11.9 mmol, 87%) as a white solid. R_f (1/1 EtOAc/heptane) = 0.22. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.41 (s, 1 H), 7.35-6.98 (m, 25 H), 5.30 (br d, *J* = 8.0 Hz, 1 H), 5.13-5.07 (m, 2 H), 4.94 (d, *J* = 10.9 Hz, 1 H), 4.90 (d, *J* = 10.9 Hz, 1 H), 4.85 (d, *J* = 10.5 Hz, 1 H), 4.64-4.49 (m, 5 H), 4.44-4.37 (m, 1 H), 4.35-4.27 (m, 3 H), 3.92 (t, *J* = 9.3 Hz, 1 H), 3.81 (t, *J* = 8.9 Hz, 1 H), 3.74-3.60 (m, 4 H), 3.69 (s, 3 H), 1.92-1.62 (m, 4 H). ^{13}C-NMR (50 MHz, $CDCl_3$) δ = 172.0, 155.7, 145.4, 138.3, 137.8, 137.7, 135.9, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.5, 127.4, 122.6, 86.6, 81.5, 79.1, 77.9, 75.3, 74.8, 74.5, 73.8, 73.1,

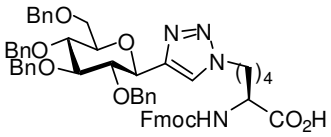
68.9, 66.8, 52.9, 52.3, 49.2, 29.2, 25.9. IR (film) ν = 3321, 3028, 2950, 2863, 2249, 1951, 1722, 1515 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{50}H_{54}N_4NaO_9$ $(M+Na)^+$ 877.3788, found 877.3805.

Fmoc-L-T1P{4-[β-D-Glc(Bn)$_4$]}-OH (24)



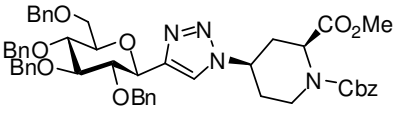
Applying general procedure A gave **24** as a white solid (626 mg, 0.695 mmol, 69%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.74 (d, *J* = 7.5 Hz, 2 H), 7.55-7.51 (m, 2), 7.39-6.94 (m 24 H), 5.01-4.33 (m, 13 H), 4.18-4.03 (m, 2 H), 3.88-3.81 (m, 5 H), 3.58-3.42 (m, 1 H). IR (film) ν = 3058, 3032, 2863, 2245, 1718 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{54}H_{52}N_4NaO_9$ $(M+Na)^+$ 923.3632, found 923.3629.

Fmoc-L-T1B{4-[β-D-Glc(Bn)$_4$]}-OH (26)



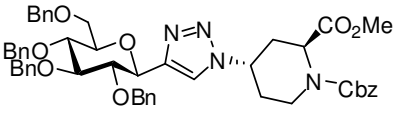
Applying general procedure A gave **26** as a white solid (662 mg, 0.702 mmol, 71%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.75 (d, *J* = 7.5 Hz, 2 H), 7.61-7.55 (m, 2 H), 7.45-6.95 (m, 25 H), 5.40 (d, *J* = 6.9 Hz, 1 H), 4.92 (dd, *J* = 24.0, 11.1 Hz, 2 H), 4.82 (d, *J* = 10.8 Hz, 1 H), 4.67 (d, *J* = 10.9 Hz, 1 H), 4.59-4.23 (m, 10 H), 4.20 (t, *J* = 6.8 Hz, 1 H), 4.01 (t, *J* = 9.5 Hz, 1 H), 3.81 (t, *J* = 8.7 Hz, 1 H), 3.75-3.53 (m, 4 H), 2.02-1.78 (m, 2 H), 1.77-1.58 (m, 2 H), 1.39-1.22 (m, 1 H), 1.14-0.96 (m, 1 H). IR (film) ν = 3058, 3021, 2924, 2859, 1714 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{57}H_{58}N_4NaO_9$ $(M+Na)^+$ 965.4101, found 965.4126.

(2*S*,4*R*)-*N*-Cbz-4-{4-[β-D-Glc(Bn)$_4$]-[1,2,3]triazol-1-yl} pipecolic acid methyl ester (28)



Applying general procedure A gave **28** (39 mg, 0.045 mmol, 49%) as a white solid. R_f (1/1 EtOAc/heptane) = 0.36. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.45 (s, 1 H), 7.39-6.95 (m, 25 H), 5.24-5.08 (m, 3 H), 4.95 (d, *J* = 11.1 Hz, 1 H), 4.91 (d, *J* = 11.2 Hz, 1 H), 4.85 (d, *J* = 10.8 Hz, 1 H), 4.66-4.47 (m, 6 H), 4.39-4.25 (m, 2 H), 3.95 (t, *J* = 9.2 Hz, 1 H), 3.84-3.61 (m, 8 H), 3.21 (dt, *J* = 25.0, 12.5 Hz, 1 H), 2.84-2.67 (m, 1 H), 2.20-2.10 (m, 2 H), 2.00-1.86 (m, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.7, 155.9, 145.8, 138.5, 138.0, 137.9, 136.1, 129.6, 128.4, 128.3, 128.3, 128.1, 128.0, 127.9, 127.7, 127.6, 127.5, 127.4, 121.8, 86.9, 81.7, 79.2, 78.1, 75.5, 75.0, 74.5, 74.0, 73.3, 69.1, 67.6, 52.8, 52.4, 52.1, 37.9, 30.9, 28.3. HRMS (ESI): *m/z* calculated for $C_{51}H_{54}N_4NaO_9$ $(M+Na)^+$ 889.3789, found 889.3815.

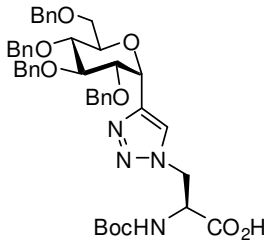
(2*S*,4*S*)-*N*-Cbz-4-{4-[β-D-Glc(Bn)$_4$]-[1,2,3]triazol-1-yl} pipecolic acid methyl ester (30)



Applying general procedure A gave **30** (42 mg, 0.049 mmol, 60%) as a white solid. R_f (1/1 EtOAc/heptane) = 0.36. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.44 (s, 1 H), 7.34-7.03 (m, 25 H), 5.17-5.14 (m, 2 H), 4.92 (d, *J* = 2.8 Hz, 2 H), 4.85 (d, *J* = 10.9 Hz, 1 H), 4.73-4.47 (m, 6 H), 4.38 (d, *J* = 11.0 Hz, 1 H), 4.00-3.59 (m, 7 H), 3.43 (s, 2 H), 2.84-2.78 (m, 1 H), 2.42-2.35 (m, 2 H), 2.19-2.11 (m, 1 H).

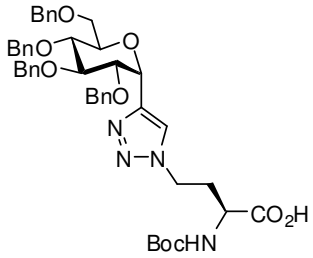
^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.7, 155.8, 145.4, 138.4, 137.9, 136.0, 129.6, 128.5, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.5, 127.4, 120.9, 86.8, 81.6, 79.3, 78.1, 75.5, 75.0, 74.7, 73.9, 73.3, 69.0, 67.9, 55.0, 54.9, 53.7, 52.7, 40.5, 32.7, 31.8. HRMS (ESI): *m/z* calculated for $C_{51}H_{54}N_4NaO_9$ $(M+Na)^+$ 889.3789, found 889.3812.

Boc-L-T1M{4-[α-D-Glc(Bn)$_4$]}-OH (37)



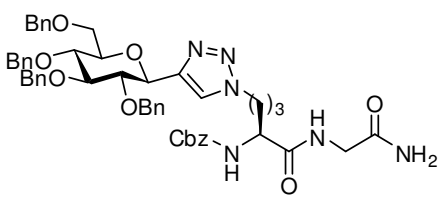
Applying general procedure A gave **37** as a white solid (10 mg, 0.01 mmol, 60%). R_f (1% AcOH in EtOAc)= 0.52. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.67 (s, 1 H), 7.31-7.09 (m, 20 H), 5.48 (br d, *J* = 4.7 Hz, 1 H), 5.29 (br d, *J*=4.9 Hz, 1 H), 4.95-4.90 (m, 2 H). 4.80-4.74 (m, 3 H), 4.68-4.58 (m, 4 H), 4.49 (d, *J* = 10.8 Hz, 1 H), 4.45 (d, *J* = 12.1 Hz, 1 H), 4.04-3.94 (m, 2 H), 3.75 (t, *J* = 8.9 Hz, 1 H), 3.67-3.55 (m, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.3, 155.2, 142.9, 138.5, 137.9, 137.8, 137.7, 129.0, 128.5, 128.3, 128.0, 127.9, 127.7, 127.6, 125.6, 125.3, 82.4, 80.7, 79.4, 78.0, 75.5, 75.1, 73.6, 73.4, 73.0, 69.2, 68.6, 54.1, 51.3, 28.6. IR (film) ν = 3404, 3032, 2915, 2859, 2358, 2336, 1709 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{44}H_{50}N_4NaO_9$ $(M+Na)^+$ 801.3475, found 801.3445. $[\alpha]_D$ = +55.2 (c = 0.48, $CHCl_3$).

Boc-L-T1E{4-[α-D-Glc(Bn)$_4$]}-OH (38)



Applying general procedure A gave **38** as a white solid (49 mg, 0.06 mmol, 60%). R_f (1% AcOH in EtOAc) = 0.53. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.64 (s, 1 H), 7.34-7.09 (m, 20 H), 5.45 (br s, 1 H), 5.28 (d, *J* = 5.5 Hz, 1 H), 4.98 (d_{AB}, *J* = 11.1 Hz, 1 H), 4.85 (d_{AB}, *J* = 10.9 Hz, 1 H), 4.80 (d_{AB}, *J* = 10.7 Hz, 1 H), 4.69 (d_{AB}, *J* = 11.7 Hz, 1 H), 4.61 (d_{AB}, *J* = 11.7 Hz, 1 H), 4.56 (d_{AB}, *J* = 12.3 Hz, 1 H), 4.49 (d_{AB}, *J* = 10.7 Hz, 1 H), 4.46 (d_{AB}, *J* = 12.1 Hz, 1 H), 4.45-4.20 (m, 3 H), 3.98 (dd, *J* = 9.4, 9.2 Hz, 1 H), 3.75-3.56 (m, 5 H), 2.47 (br s, 2 H), 1.44 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 155.4, 143.3, 138.6, 137.6, 137.4, 128.3, 128.1, 127.9, 127.7, 127.6, 124.8, 82.4, 80.4, 79.7, 78.4, 75.5, 75.1, 73.6, 73.4, 72.5, 69.2, 69.0, 46.3, 32.8, 30.0, 28.7. IR (film) ν = 3408, 3023, 2976, 2872, 2353, 2332, 1705 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{45}H_{53}N_4O_9$ $(M+H)^+$ 793.3812, found 793.3836. $[\alpha]_D$ = +60.0 (c = 0.31, $CHCl_3$).

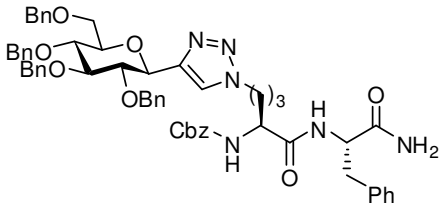
Cbz-L-T1P{4-[β-D-Glc(Bn)$_4$]}-Gly-NH$_2$ (39)



Applying general procedure C gave **39** (361 mg, 82%). Applying general procedure B gave **39** (22 mg, 96%). R_f (2/1 EtOAc/heptane) = 0.17. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.73 (s, 1 H), 7.33-6.95 (m, 25 H), 5.10 (d, *J* = 12.3 Hz, 1 H), 5.09 (d, *J* = 12.3 Hz, 1 H), 4.91 (s, 2 H), 4.85 (d, *J* = 10.9 Hz, 1 H), 4.61-4.50 (m, 4 H), 4.47 (d, *J* = 12.0 Hz, 1 H), 4.34 (d, *J* = 7.0 Hz, 2 H), 4.23 (d, *J* = 11.0 Hz, 1 H), 4.14 (dd, *J* = 5.4, 8.4 Hz, 1 H), 3.91-3.70 (m, 7 H), 3.63-3.60 (m, 1 H), 2.02-1.60 (m, 4 H). ^{13}C-NMR (50 MHz. $CDCl_3$/MeOD) δ = 173.0. 172.5. 157.1. 145.6. 138.3. 137.8. 137.7. 137.6. 136.2.

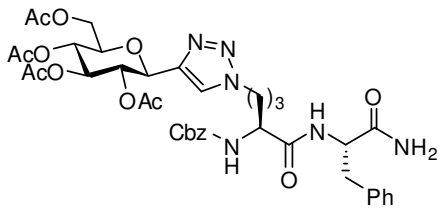
128.3. 128.3. 128.3. 128.2. 128.0. 127.9. 127.8. 127.8. 127.7. 127.7. 127.6. 127.6. 127.5. 123.6. 86.7. 81.7. 79.1. 78.0. 75.5. 75.0. 74.6. 73.8. 73.3. 68.8. 66.9. 54.5. 42.0. 28.5. 26.3. IR (film) ν = 3343, 2945, 2824, 2483, 2232, 2072 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{51}H_{56}N_6O_9Na$ $(M+Na)^+$ 919.40065, found 919.39922.

Cbz-L-T1P{4-[β-D-Glc(Bn)$_4$]}-L-Phe-NH$_2$ (40)



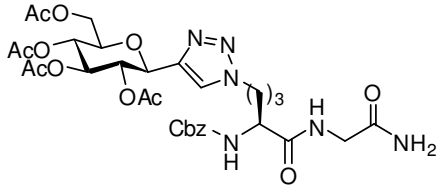
Applying general procedure C gave **40** (418 mg, 86%). Applying general procedure B gave **40** (21 mg, 87%). R_f (2/1 EtOAc/heptane) = 0.20. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.71 (s, 1 H), 7.33-6.93 (m, 30 H), 5.06 (d, *J* = 12.2 Hz, 1 H), 5.03 (d, *J* = 12.2 Hz, 1 H), 4.89 (s, 2 H), 4.83 (d, *J* = 10.9 Hz, 1 H), 4.62-4.48 (m, 5 H), 4.44 (d, *J* = 11.9 Hz, 1 H), 4.25 (t, *J* = 7.0 Hz, 2 H), 4.24 (d, *J* = 10.9 Hz), 4.03 (dd, *J* = 5.8, 8.0 Hz, 1 H), 3.85-3.76 (m, 2 H), 3.73-3.66 (m, 3 H), 3.62-3.58 (m, 1 H), 3.16 (dd, *J* = 5.8, 14.0 Hz, 1 H), 2.90 (dd, *J* = 8.4, 14.0 Hz, 1 H), 1.78-1.48 (m, 4 H). ^{13}C-NMR (75 MHz, $CDCl_3$/MeOD) δ = 174.4, 172.4, 157.3, 146.1, 138.8, 138.3, 138.2, 138.1, 137.0, 136.5, 129.5, 128.8, 128.8, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 127.2, 123.8, 87.2, 82.2, 79.6, 78.4, 76.0, 75.4, 75.1, 74.3, 73.8, 69.3, 67.4, 54.8, 54.3, 50.0, 37.9, 29.2, 26.3. IR (film) ν = 3369, 3282, 2945, 2828, 1696, 1645 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{58}H_{62}N_6O_9Na$ $(M+Na)^+$ 1009.44760, found 1009.44631.

Cbz-L-T1P{4-[β-D-Glc(Ac)$_4$]}-L-Phe-NH$_2$ (43)



Applying general procedure B gave **43** (18 mg, 88%). R_f (EtOAc) = 0.42. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.71 (s, 1 H), 7.36-7.16 (m, 10 H), 5.37 (t, *J* = 9.4 Hz, 1 H), 5.23 (t, *J* = 9.7 Hz, 1 H), 5.19 (t, *J* = 9.7 Hz, 1 H), 5.09 (d, *J* = 12.1 Hz, 1 H), 5.05 (d, *J* = 12.1 Hz, 1 H), 4.75 (d, *J* = 10.0 Hz, 1 H), 4.63 (dd, *J* = 8.1, 14.3 Hz, 1 H), 4.37-4.21 (m, 3 H), 4.14 (dd, *J* = 2.0, 12.5 Hz, 1 H), 4.12-4.05 (m, 1 H), 3.91 (ddd, *J* = 2.0, 4.8, 12.4 Hz, 1 H), 3.17 (dd, *J* = 6.3 14.4 Hz, 1 H), 2.94 (dd, *J* = 8.2, 13.8 Hz, 1 H), 2.07 (s, 3 H), 2.06 (s, 3 H), 2.02 (s, 3 H), 1.88 (s, 3 H), 1.84-1.74 (m, 2 H), 1.68-1.59 (m, 1 H), 1.54-1.43 (m, 1 H). ^{13}C-NMR (50 MHz, $CDCl_3$/MeOD) δ = 173.5, 171.5, 170.9, 170.2, 169.9, 169.7, 143.9, 136.3, 135.7, 128.9, 128.2, 128.2, 128.0, 127.7, 126.6, 122.6, 75.8, 73.6, 72.8, 71.4, 68.1, 66.9, 61.9, 54.0, 53.5, 49.5, 37.2, 25.7, 20.3, 20.2, 20.0. IR (KBr) ν = 3307, 2955, 1753, 1675, 1533 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{38}H_{46}N_6O_{13}Na$ $(M+Na)^+$ 817.30205, found 817.29744.

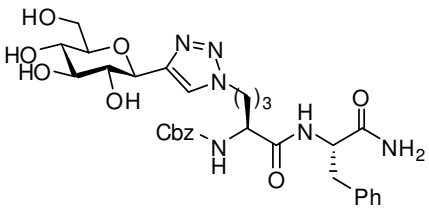
Cbz-L-T1P{4-[β-D-Glc(Ac)$_4$]}-Gly-NH$_2$ (45)



Applying general procedure B gave **45** (11 mg, 62%). R_f (EtOAc) = 0.11. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.71 (s, 1 H), 7.33-7.28 (m, 5 H), 5.33 (t, *J* = 9.4 Hz, 1 H), 5.20 (t, *J* = 9.6 Hz, 1 H), 5.15 (t, *J* = 9.6 Hz, 1 H), 5.09 (d, *J* = 12.2 Hz, 1 H), 5.05 (d,

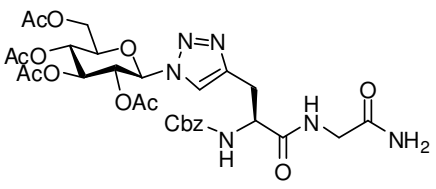
J = 12.3 Hz, 1 H), 4.72 (d, *J* = 10.0 Hz, 1 H), 4.44-4.29 (m, 1 H), 4.26 (dd, *J* = 12.4, 4.8 Hz, 1 H), 4.17-4.08 (m, 1 H), 4.11 (dd, *J* = 12.5, 1.9 Hz, 1 H), 3.94-3.75 (m, 2 H), 3.88 (ddd, *J* = 9.6, 4.7, 2.0 Hz, 1 H), 2.05 (s, 3 H), 2.04 (s, 3 H), 1.99 (s, 3 H), 1.98-1.90 (m, 2 H), 1.85 (s, 3 H), 1.82-1.71 (m, 1 H), 1.63-1.51 (m, 1 H). ^{13}C-NMR (75 MHz, $CDCl_3$/MeOD) δ = 172.3, 171.9, 170.8, 170.2, 169.9, 169.7, 156.7, 144.2, 135.8, 128.2, 128.0, 127.7, 122.7, 75.9, 73.6, 72.8, 71.4, 68.2, 66.9, 66.7, 62.0, 54.1, 42.0, 30.5, 28.5, 26.0, 20.3, 20.2, 20.1. IR (KBr) ν = 3351, 2953, 1754, 1675, 1525 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{31}H_{40}N_6O_{13}Na$ $(M+Na)^+$ 727.25510, found 727.25131.

Cbz-L-T1P[4-(β-D-Glc)]-L-Phe-NH$_2$ (46)



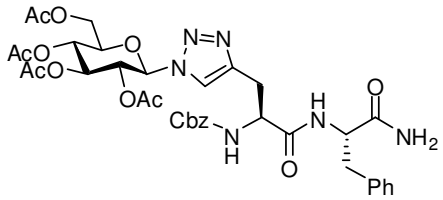
To a solution of glucodipeptide **43** (12 mg, 15 μmol) in MeOH (2 mL) was added K_2CO_3 (0.29 mg, 1.5 μmol). The reaction mixture was stirred overnight at ambient temperature. The reaction mixture was neutralized with acidic Amberlite and stirring was continued for another 10 min. The product **46** was isolated after filtration and evaporation of solvent as a white solid (8.9 mg, 95%). ^{1}H-NMR ($CDCl_3$/MeOD, 400 MHz) δ = 7.86 (s, 1 H), 7.35-7.30 (m, 5 H), 7.26-7.16 (m, 5 H), 5.10 (d, *J* = 12.7 Hz, 1 H), 5.06 (d, *J* = 12.0 Hz, 1 H), 4.62 (dd, *J* = 8.6, 5.8 Hz, 1 H), 4.41 (d, *J* = 8.6 Hz, 2 H), 4.33 (t, *J* = 6.8 Hz, 2 H), 4.06 (dd, *J* = 7.9, 5.8 Hz, 1 H), 3.88 (d, *J* = 11.3 Hz, 1 H), 3.72 (dd, *J* = 12.1, 4.7 Hz, 1 H), 3.60-3.43 (m, 4 H), 3.18 (dd, *J* = 14.0, 5.8 Hz, 1 H), 2.92 (dd, *J* = 14.0, 8.8 Hz, 1 H), 1.86-1.75 (m, 2 H), 1.68-1.49 (m, 2 H). ^{13}C-NMR ($CDCl_3$/MeOD, 75 MHz) δ = 173.7, 171.7, 156.4, 136.1, 135.6, 128.4, 127.6, 127.5, 127.3, 127.0, 125.9, 122.4, 79.9, 76.5, 73.5, 72.9, 69.4, 66.1, 61.0, 53.8, 53.3, 48.9, 36.7, 27.9, 25.2. HRMS (ESI) *m/z* calculated for $C_{30}H_{38}N_6O_9Na$ $(M+Na)^+$ 649.25980, found 649.26012.

Cbz-L-T4M{1-[β-D-Glc(Ac)$_4$]}-Gly-NH$_2$ (49)



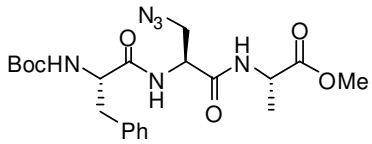
Appying general procedure B gave **49** (18 mg, 99%). R_f (EtOAc) = 0.52. ^{1}H-NMR ($CDCl_3$/MeOD, 400 MHz) δ = 7.93 (s, 1 H), 7.42-7.25 (m, 5 H), 5.99-5.90 (m, 1 H), 5.53 (s, 1 H), 5.50-5.45 (m, 2 H), 5.34-5.23 (m, 1 H), 5.11 (d, *J* = 12.4 Hz, 1 H), 5.08 (d, *J* = 12.4 Hz, 1 H), 4.49 (t, *J* = 6.2, 6.2 Hz, 1 H), 4.32 (dd, *J* = 12.6, 4.8 Hz, 1 H), 4.18 (dd, *J* = 12.6, 2.1 Hz, 1 H), 4.16-4.10 (m, 1 H), 3.92 (d, *J* = 17.1 Hz, 1 H), 3.84 (d, *J* = 17.0 Hz, 1 H), 3.29-3.18 (m, 2 H), 2.09 (s, 1 H), 2.08 (s, 1 H), 2.04 (s, 1 H), 1.83 (s, 1 H). ^{13}C-NMR (75 MHz, $CDCl_3$/MeOD) δ = 172.1, 171.5, 170.5, 169.7, 169.3, 168.9, 156.3, 135.6, 127.9, 127.6, 127.4, 121.6, 85.0, 74.2, 72.1, 70.2, 67.3, 66.6, 61.1, 54.1, 41.8, 27.1, 19.7, 19.6, 19.1. IR (KBr) ν = 3292, 2948, 1753, 1687, 1535 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{29}H_{36}N_6O_{13}Na$ $(M+Na)^+$ 699.22380, found 699.22129.

Cbz-L-T4M{1-[β-D-Glc(Ac)$_4$]}-L-Phe-NH$_2$ (50)



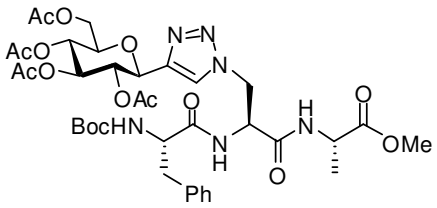
Appying general procedure B gave **50** (18 mg, 92%). R_f (EtOAc) = 0.60. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.74 (s, 1 H), 7.41-7.16 (m, 10 H), 5.96-5.87 (m, 1 H), 5.53 (s, 1 H), 5.49-5.41 (m, 2 H), 5.06 (s, 2 H), 4.63 (dd, *J* = 8.1, 5.9 Hz, 1 H), 4.42 (t, *J* = 6.4, 6.4 Hz, 1 H), 4.31 (dd, *J* = 12.6, 4.9 Hz, 1 H), 4.16 (dd, *J* = 12.6, 2.1 Hz, 1 H), 4.11 (ddd, *J* = 10.2, 4.8, 2.1 Hz, 1 H), 3.20-3.15 (m, 2 H), 3.05 (dd, *J* = 15.2, 6.9 Hz, 1 H), 2.96 (dd, *J* = 13.9, 8.3 Hz, 1 H), 2.09 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 3 H), 1.82 (s, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$/MeOD) δ = 173.6, 170.7, 170.5, 169.8, 169.4, 168.9, 156.1, 143.1, 136.2, 135.6, 128.6, 128.5, 128.0, 127.7, 127.4, 126.3, 121.4, 85.0, 74.3, 72.2, 70.1, 67.3, 66.6, 61.2, 53.9, 53.6, 36.8, 27.2, 19.8, 19.7, 19.2. IR (KBr) ν = 3351, 2942, 1748, 1693, 1666, 1524 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{36}H_{42}N_6O_{13}Na$ $(M+Na)^+$ 789.27075, found 789.26751.

Boc-L-Phe-L-Dap(N$_3$)-L-Ala-OMe (51)



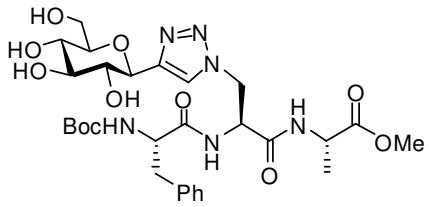
To a solution of Boc-L-Dap(N_3)-OH (0.50 g, 2.2 mmol) and L-Ala-OMe (328 mg, 2.28 mmol) in CH_2Cl_2 (25 mL) at 0 °C was added DIPEA (561 mg, 4.34 mmol), HOBt (323 mg, 2.39 mmol) and was stirred for 5 min before adding EDCI (458 mg, 2.39 mmol) very slowly. The reaction was stirred at 0 °C for 30 min and then at rt over night. The reaction was diluted with EtOAc (50 mL), washed with saturated $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated. The product was purified by column chromatography (5% MeOH in DCM) to obtain dipeptide Boc-L-Dap(N_3)-L-Ala-OMe (596 mg, 1.89 mmol). R_f (EtOAc/heptane 2:1) = 0.68. ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 6.83-6.82 (bd, 1 H), 5.26 (bs, 1 H), 4.59 (q, *J* = 7.2 Hz , 1 H), 4.28 (bs, 1 H), 3.88 (dd, *J* = 4.2, 12.3 Hz, 1 H), 3.54-3.50 (m, 1 H), 1.47 (s, 9 H), 1.44 (d, *J* = 7.2 Hz, 3 H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 172.4, 168.5, 154.8, 53.2, 52.1, 51.7, 47.9, 27.8, 17.8; HRMS (ESI) *m/z* calculated for $C_{12}H_{22}N_5O_5$ $(M+H)^+$ 316.1621, found 316.1627. Boc-L-Dap(N_3)-L-Ala-OMe was dissolved in TFA/CH_2Cl_2 (1:1 10 mL). After 20 min the solvent was removed by evaporation The desired product was obtained sufficiently pure in a quantitative way. R_f (EtOAc/heptane 2:1) = 0.21. The same procedure as the first part of this synthesis was used for the coupling with Boc-Phe-OH. After purification by gradient flash chromatography (EtOAc/heptane) the desired product was obtained (596 mg, 1.29 mmol, 63%); R_f (EtOAc/heptane 2:1) = 0.53. IR (film) ν = 3300, 3062, 2977, 2924, 2106, 1745, 1692, 1649, 1526 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.35-7.20 (m, 5 H), 6.95 (br s, 1 H), 6.73 (d, *J* = 7.8 Hz, 1 H), 6.42 (d, *J* = 6.3 Hz, 1 H), 4.58-4.49 (m, 2 H), 4.37 (dd, *J* = 6.5, 13.3 Hz, 1 H), 3.88 (m, 1 H), 3.75 (s, 3 H), 3.44 (dd, *J* = 5.6, 12.3 Hz, 1 H), 3.15 (dd, *J* = 6.0, 14.1 Hz, 1 H), 3.06 (dd, *J* = 7.6, 14.1 Hz, 1 H), 1.41 (d, J = 7.2 Hz, 3 H), 1.41 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 138.1, 129.1, 128.8, 127.2, 80.7, 77.4, 77.0, 76.6, 58.0, 52.5, 52.2, 51.8, 48.4, 37.8, 28.2, 17.8. HRMS (ESI) *m/z* calculated for $C_{21}H_{31}N_6O_6$ $(M+H)^+$ 463.2305, found 463.2328.

Boc-L-Phe-L-T1M{4-[β-D-Glc(Ac)$_4$]}-L-Ala-OMe (52)



Applying general procedure B (adjusted to 13 mg **51** (28 µmol) and 10 mg **8** (28 µmol)) afforded the desired product **52** (21 mg, 26 µmol, 91%). R_f (5% MeOH in DCM) = 0.34. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.67 (s, 1 H), 7.34-7.19 (m, 5 H), 7.10-7.06 (m, 2 H), 5.35 (t, *J* = 9.4 Hz, 1 H), 5.19 (t, *J* = 9.7 Hz, 1 H), 5.15 (t, *J* = 9.7 Hz, 1 H), 5.02 (d, *J* = 6.0 Hz, 1 H), 4.96-4.91 (m, 1 H), 4.86 (dd, *J* = 4.7, 13.7 Hz, 1 H), 4.72-4.65 (m, 2 H), 4.42 (p, *J* = 7.2 Hz, 1 H), 4.33-4.24 (m, 2 H), 4.11 (dd, *J* = 2.1, 12.4 Hz, 1 H), 3.85 (ddd, *J* = 2.1, 4.9, 10.0 Hz, 1 H), 3.74 (s, 3 H), 3.19 (dd, *J* = 5.4, 14.1 Hz, 1 H), 2.96 (dd, *J* = 8.6, 14.1 Hz, 1 H), 2.06 (s, 3 H), 2.05 (s, 3 H), 2.01 (s, 3 H), 1.38 (s, 9 H), 1.35 (d, *J* = 7.3 Hz, 3 H).

Boc-L-Phe-L-T1M[4-(β-D-Glc)]-L-Ala-OMe (53)



Applying general procedure B, (adjusted to 13 mg **51** (28 µmol) and 5.4 mg **9** (28 µmol)) afforded the desired product **53** (9 mg, 14 µmol, 50%). R_f (10% MeOH in DCM) = 0.12. ^{1}H-NMR (300MHz, MeOD) δ = 7.98 (s, 1 H), 7.29-7.18 (m, 5 H), 4.72-4.54 (m, 1 H), 4.22 (dd, *J* = 9.8, 5.1 Hz, 1 H) , 3.85-3.81 (m, 1 H), 3.73 (s, 3 H), 3.66-3.62 (m, 1 H), 3.56-3.49 (m, 1 H), 3.48-3.44 (m, 1 H), 3.42-3-39 (m, 2 H), 3.09-2.71 (m, 2 H), 1.38 (d, *J* = 7.3 Hz, 3 H), 1.35 (s, 9 H).

3.7 References

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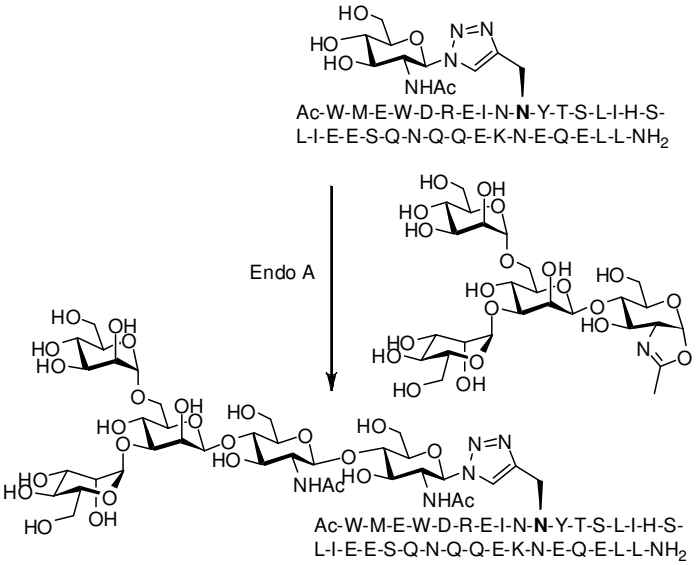
4 Chemoenzymatic glycosylation of triazole-linked glycopeptides

Abstract

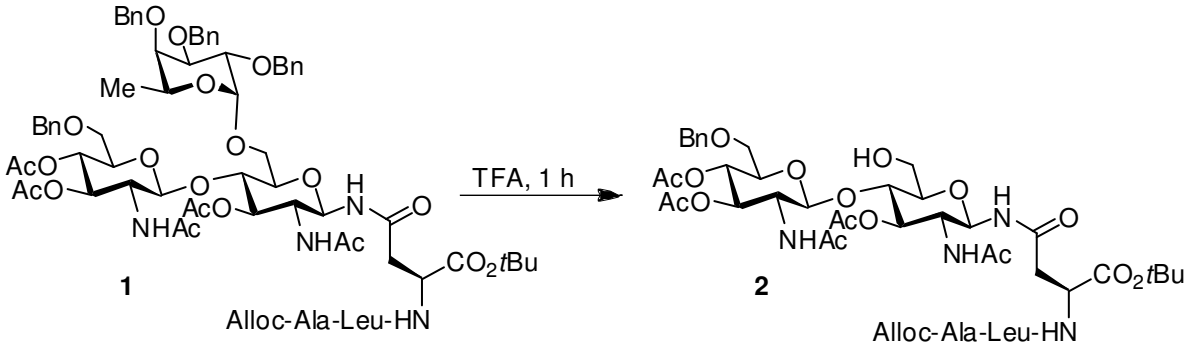
Triazole-linked *C*- and *N*-glycodipeptides were enzymatically glycosylated with a carbohydrate oxazoline donor under the action of the enzyme Endo A. Although the conversion of the *C*-triazolyl glycopeptide stopped at a conversion of 50%, the corresponding *N*-triazolyl derivative was converted to the pentasaccharide with an efficiency of 80%. Next, the same chemoenzymatic glycosylation procedure was applied to a triazole-linked *N*-GlcNAc-modified peptide C34, of the C-terminal fragment of HIV-1 gp41. The resulting novel triazole-linked glycopeptide appeared fully resistant to peptide *N*-glycanase (PNGase F), a serum peptidase that effectively digests the native amide-linked glycopeptide.



Part of the research described in this chapter was performed in the research group of Prof. Dr. Lai-Xi Wang, Institute of Human Virology, Department of Biochemistry and Molecular Biology, University of Maryland.

4.1 Introduction

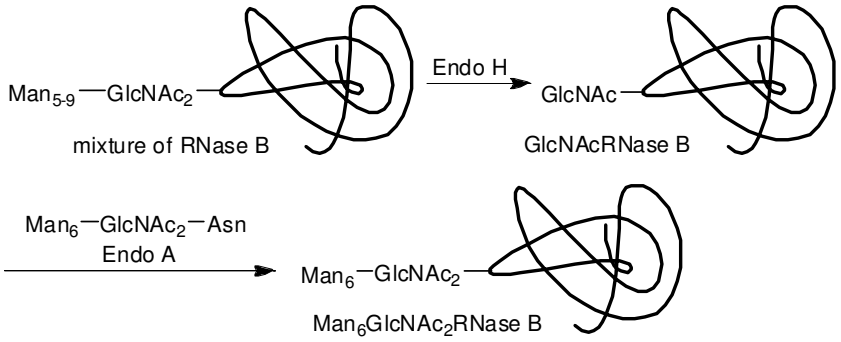
Methods for the chemical synthesis of glycopeptides have advanced considerably, and peptides carrying relatively simple saccharides can now be prepared by a variety of procedures.[1] Most commonly preformed Fmoc-protected glycoamino acid building blocks are employed in the stepwise solid phase assembly of the peptide backbone, since the very nature of the glycosidic bond-forming reaction conditions often hamper the side-chain glycosylation of preformed full-length peptides and is not suitable for the synthesis of *O*-linked glycopeptides. A disadvantage of early introduction of oligosaccharides onto the peptide, however, is that *O*–glycosidic linkages are known to be labile towards the harsh acidic conditions often used in peptide synthesis (Scheme 4.1),[2] or can be subject to β-elimination under basic conditions. A range of alternative techniques have also been developed for the synthesis of glycoproteins, *e.g.* native chemical ligation and suppressor tRNA technology, but have not yet matured to the level of suitability for the synthesis of glycoproteins containing complex glycans.



Scheme 4.1 Acidic lability of an α-fucosidic linkage in **1**

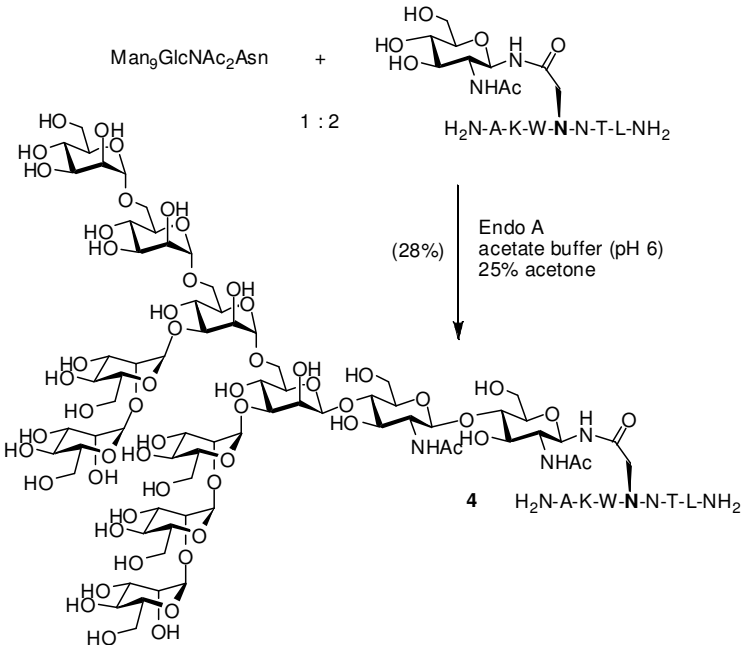
Given the limitations pertaining to current state-of-the-art for synthesis of complex glycoproteins, an interesting alternative involves the chemical elongation of a growing peptide chain with a chemically inert amino acid building block carrying only a single sugar. Subsequently, after cleavage from the resin and purification, the glycopeptide can be conjugated to the full glycan. A promising possibility to achieve such a saccharide expansion is the chemoenzymatic approach to glycoprotein synthesis.[3,4] This is in particular true when larger and more complex carbohydrate moieties have to be incorporated, since a preformed oligosaccharide can be

incorporated in one single chemoenzymatic step, which is in contrast to a chemical incorporation where only one monosaccharide moiety at a time is added. The most commonly employed enzyme to couple complex glycans to peptides is *endo*-β-*N*-acetylglucosaminidase, an enzyme that normally hydrolyzes the chitobiose (GlcNAc–(β1→4)-GlcNAc) core of *N*–linked glycoproteins to produce a free oligosaccharide and a protein possessing a single GlcNAc at the reaction site. For example, trimming of natural glycoproteins followed by subsequent introduction of alternative carbohydrate fragments lies at the core of so-called glycoprotein remodeling. Importantly, it has been shown that several members of the family of glucosaminidases, especially *endo*–β–*N*–acetylglucosaminidase A (Endo A) from *Anthrobacter protophormiae* and *endo*–β–*N*–acetylglucosaminidase M (Endo M) from *Mucor hiemalis*, can also catalyze transglycosylation, *i.e.* the transfer of an oligosaccharide from GlcNAc to another sugar residue. Consequently, if the acceptor sugar is already attached to a peptide, the result of such a transglycosylation is a glycopeptide bearing a complex *N*-linked glycan. An impressive application of glycoprotein remodeling is the synthesis of a homogeneous glycoform of RNase B (124 amino acid residues).[5] Following treatment of a mixture of RNase B glycoforms with Endo H to produce a RNase containing a single GlcNAc residue, the glycopeptide was treated with Endo A and $(Man)_6GlcNAc_2Asn$ (obtained from ovalbumin)[6] to afford $(Man)_6(GlcNAc)_2$RNase B (Scheme 4.2). The yield of these reactions is low, and although it was shown that the presence of organic solvents could increase the synthetic utility of the process,[7] a huge excess of one of the reactants is necessary to obtain moderate yields of the desired product.



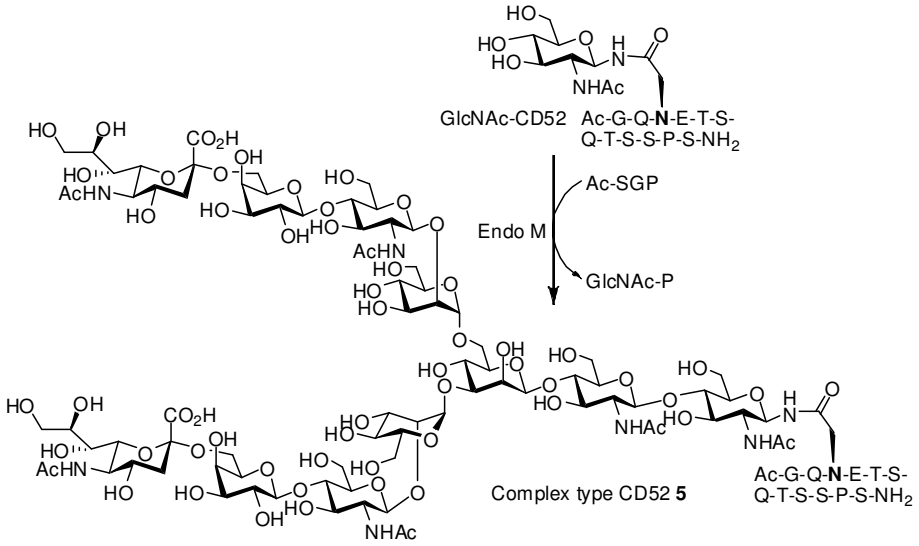
Scheme 4.2 Glycopeptide remodeling of RNase B

Wang *et al.* applied this Endo A-mediated transglycosylation to a peptide sequence of gp120 (residues 336-342), which is an envelope glycoprotein part of HIV-1 (**4**, Scheme 4.3).[8] In addition to the use of Endo A for the incorporation of high mannose type *N*-glycan, Wang *et al.* showed the transglycosylation of a biantennary sialylglycopeptide (Ac-SGP; Ac-Lys–Val–Ala–Asn[(NeuAc–Gal–GlcNAc–Man)$_2$–Man–GlcNAc$_2$]–Lys–Thr–OH)[9] to form complex type CD52 **5,** by applying enzyme Endo M (Scheme 4.4).[10]

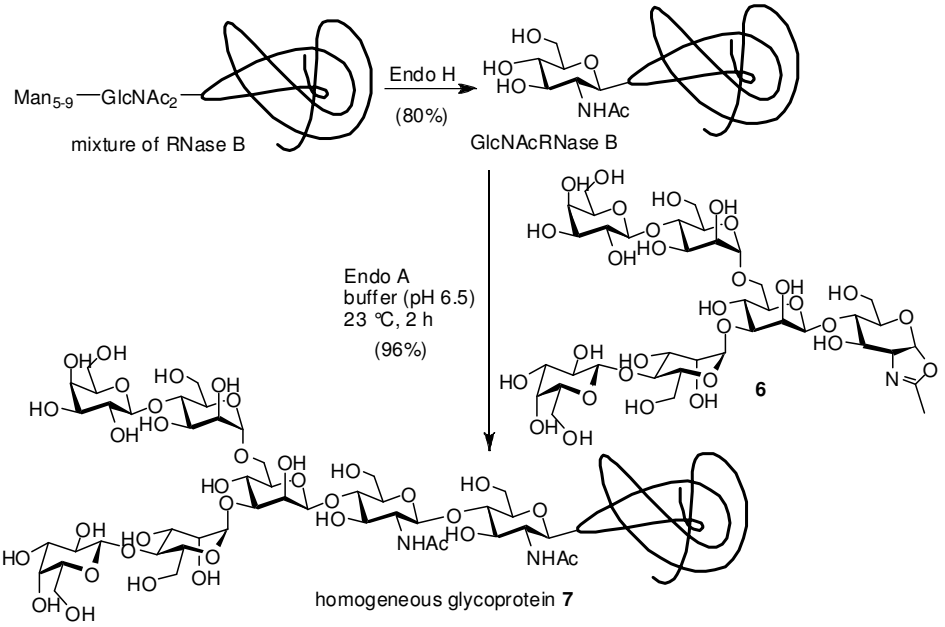


Scheme 4.3 Chemoenzymatic synthesis of Man$_9$–glycopeptide **4**

A significant improvement to the oligosaccharide transfer of glycoprotein remodeling was developed utilizing sugar oxazolines as the donor component. The oxazolines, which were designed based on putative transition state mimetics, greatly enhance the rate of transglycosylation.[11] This results in high product yields with only a small excess (ca. 3-fold) of the sugar donor. Using this method, Wang and coworkers were able to efficiently synthesize a homogeneous glycoform of RNase B in high yield (Scheme 4.5),[12] which is a significant improvement compared to the original publication by Takegawa *et al.* (Scheme 4.2).[5]



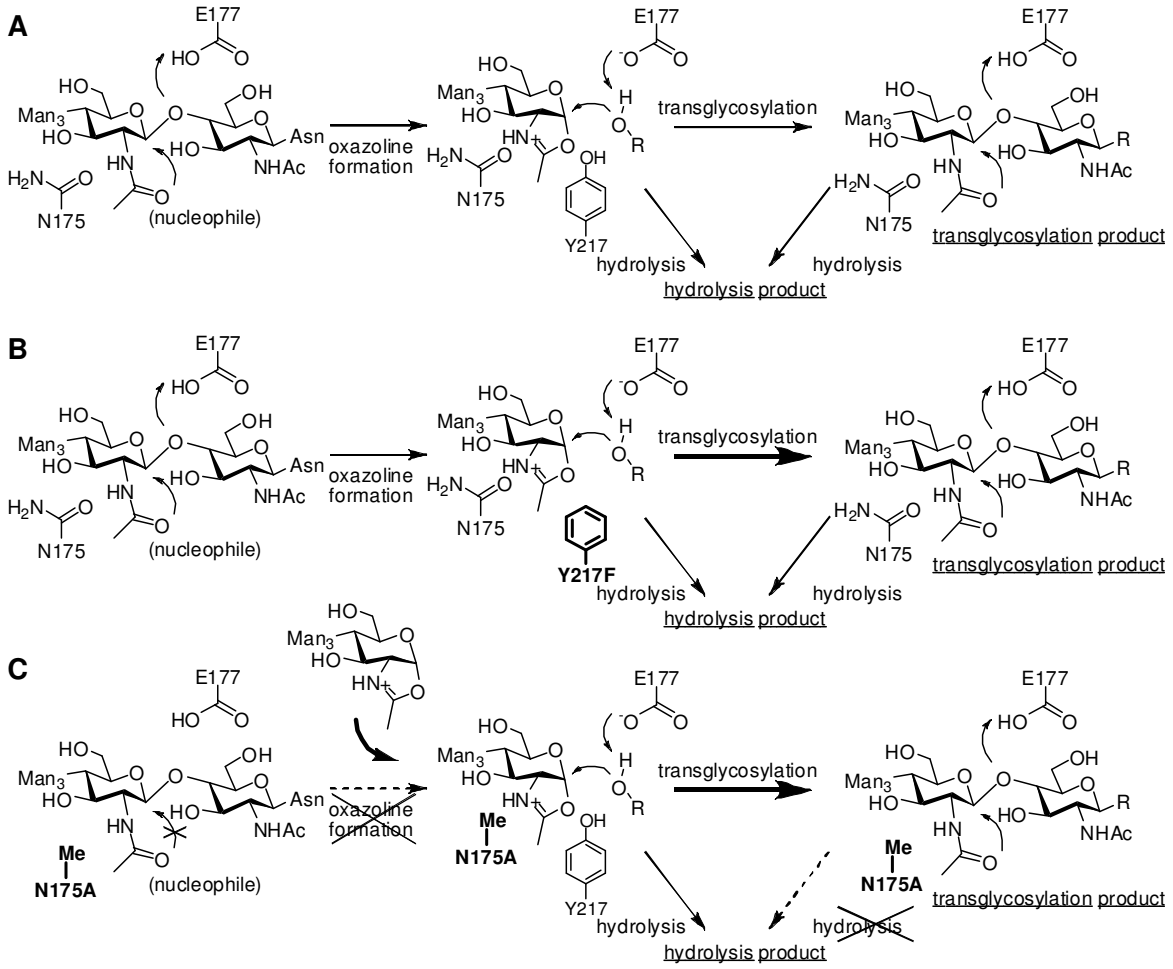
Scheme 4.4 Chemoenzymatic synthesis of complex type CD52 **5**



Scheme 4.5 Efficient chemoenzymatic synthesis of homogeneous glycoform RNase B (**7**) using the oligosaccharide oxazoline **6**

The efficient method using carbohydrate oxazolines was applied in bisglycosylation of the peptide domain HIV–1 V3, containing two N–glycans.[13] Furthermore, the technique was applied in probing the substrate structural requirement. It was revealed that the minimum substrate structure required for Endo A-catalyzed transglycosylation is a Man-(β1→4)-GlcNAc oxazoline moiety, although the enzyme

can tolerate modifications at the 3'- and/or 6'-positions of the disaccharide oxazoline, thereby allowing the transfer of both larger and selectively modified oligosaccharide moieties to the peptide acceptor.[14]



Scheme 4.6 Schematic models of the plausible mechanism of endo M catalysis.
A, wild-type; **B**, enhanced transglycosylation mutant Y217F; **C**, glycosynthase-like mutant N175A. The Glu177 residue acts as the catalytic proton donor to activate the glycosidic bond, while the Asn175 residue assists the essential orientation of the 2–acetamido group for nucleophilic attack at the activated anomeric center, leading to oxazolinium ion formation. The Tyr217 residue is important for the recognition of acceptors or water molecule activated by the Glu177 residue. The mutation of Y217F facilitates the approach of an acceptor to promote the transglycosylation and/or the exclusion of water to diminish the hydrolysis (**B**). The N175A mutant displays a loss of oxazoline formation ability; therefore, the mutant is inactive for *N*–glycan, but utilizes sugar oxazoline for transglycosylation without hydrolysis of the product (**C**).

Recently, Wang *et al.* reported that mutants of *Mucor hiemalis endo*-β-*N*-acetylglucosaminidase (Endo M) show enhanced transglycosylation and glycosynthase-like activities.[15] One mutant (Y217F) possessed much enhanced

transglycosylation activity and yet much diminished hydrolytic activity in comparison with the wild-type Endo M. Another mutant (N175A) acted like a glycosynthase. It was also found that the hydrolytic activity was knocked out, but that the mutant was able to take the highly active sugar oxazolines as donor substrates. The glycosynthase derived from *endo*-β-*N*-acetylglucosaminidases acts via a substrate-assisted mechanism (Scheme 4.6).

In 2005 Wang *et al.* published a chemoenzymatic synthesis of the glycopeptide C34 derived from HIV-1 gp41 (Figure 4.1).[16] The GlcNAc-C34 domain was transglycosylated with a Man_9 unit using Endo A (Scheme 4.7).

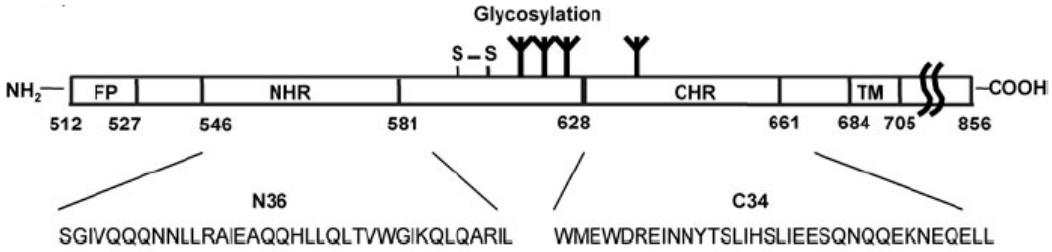
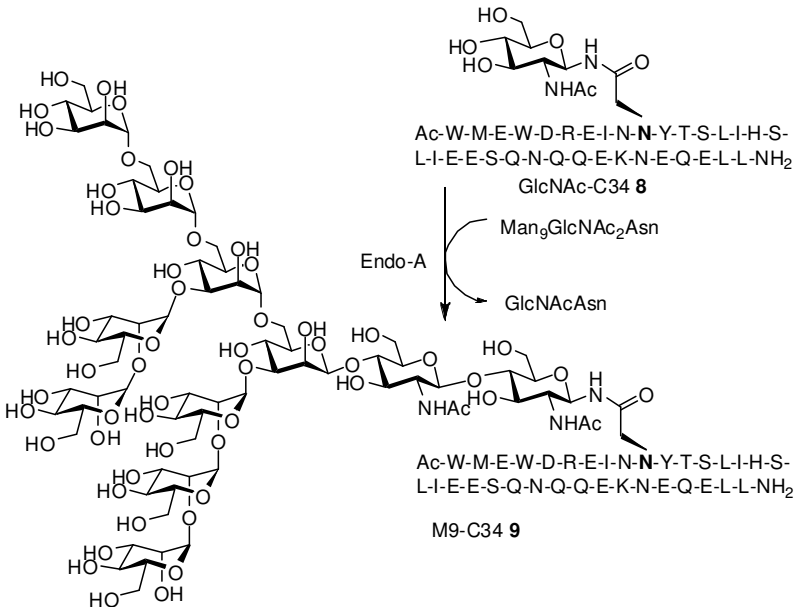


Figure 4.1 Schematic depiction of HIV-1 gp41.
The glycosylation sites, the amino acid sequences of N36 and C34, and their locations in the gp41 ectodomain are shown. FP, fusion peptide; NHR, the N-terminal heptad repeat; CHR, the C-terminal heptad repeat; TM, the transmembrane domain



Scheme 4.7 Transglycosylation in the synthesis of M_9-C34

The anti-HIV activity of the synthetic C34 glycopeptide was measured by means of a cell-fusion assay, and demonstrated potent inhibitory activities against HIV-1 infection at nanomolar concentration (IC_{50} = 7.7 nM). Although the inhibitory activity of the synthetic C34 glycopeptide was slightly decreased with respect to C34 (IC_{50} = 1.1 nM), the glycosylated C34 might still be superior to C34 in two aspects. Firstly, glycosylated C34 has a much better solubility and secondly, because of the general protective effect of glycosylation, the glycopeptides could be more resistant to protease digestion than C34, although the overall efficiency of glyco-C34 and C34 has yet to be tested in animal models.

It would therefore be of great interest to mimic these C34 glycopeptides by replacement of the amide linker between the peptide and the *N*-glycan, by our triazole linker (Figure 4.2). First of all it would be a synthetic challenge, but moreover it may answer some fundamental questions with respect to the biological activity and resistance to protease and peptide *N*-glycanase digestion[17] of isosteric replacement of the amide bond in glycoproteins with a triazole.

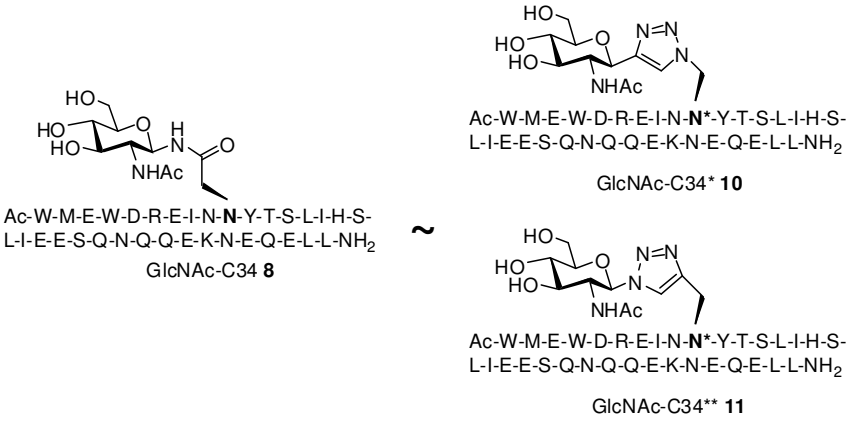
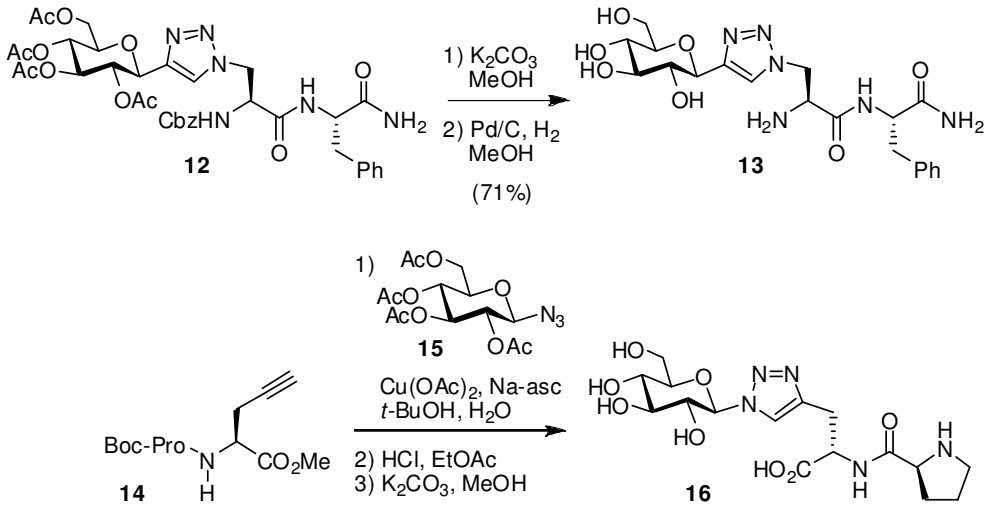


Figure 4.2 Isosteric replacement of the native amide functionality by a triazole linker

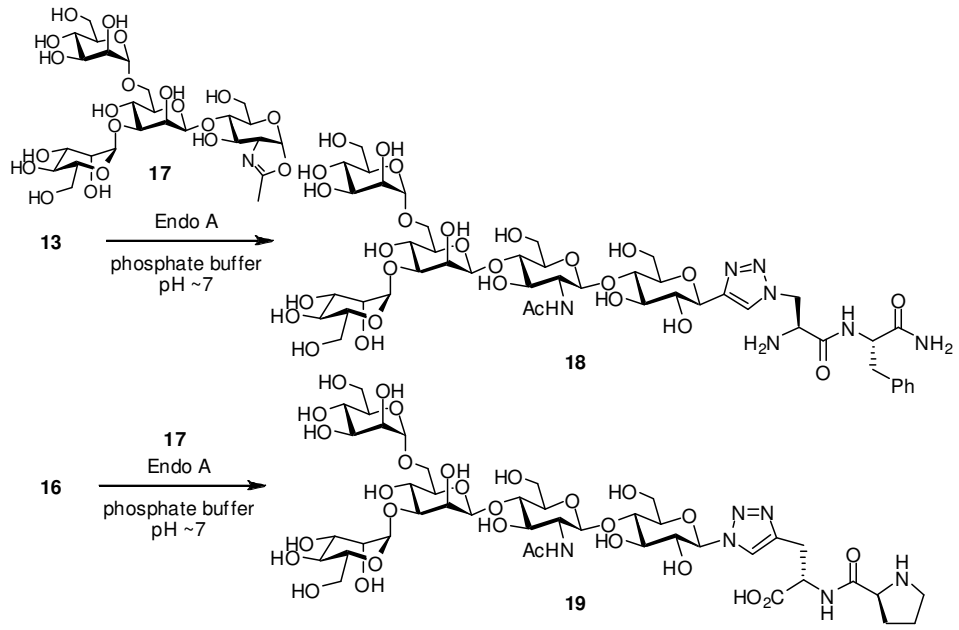
4.2 Chemoenzymatic glycosylation

Before the chemoenzymatic transglycosylation to the full-length glyco-triazole-C34 peptide was investigated, it was decided to first evaluate whether the triazole-linked glycopeptides are good substrates for chemoenzymatic transglycosylations. To this end, triazole–linked *C*-glycodipeptide **12** (Chapter 6)[18,19] was deprotected first, as depicted in Scheme 4.8, in an overall yield of 71%. Furthermore, an *N*–linked glycoamino acid coupled to L-proline on the *N*–terminus was synthesized as a

substrate for transglycosylation (Scheme 4.8).[20] It must be noted that both glycopeptides differ not only in amino acid constitution, but are also differentiated in an isosteric sense, since compound **13** belongs to the class of *C*-triazole-linked glycopeptides, while compound **16** has the carbohydrate fragment attached to N^1 of the triazole moiety.



Scheme 4.8 Deprotection of glycopeptide **12**



Scheme 4.9 Glycosylation of glucose dipeptides **13** and **16** with sugar oxazoline **17**

The fact that both glycopeptides **13** and **16** do not carry a glucosamine sugar, but a β-linked glucose instead, may also shed further light on the substrate promiscuity of Endo A. For practical reasons, the hexasaccharide oxazoline **6** was not used as the donor, but the more easily accessible tetrasaccharide Man_3-GlcNAc-oxazoline **17**,[11] which was added in threefold excess (Scheme 4.9).

Figure 4.3 shows the conversion as followed by HPLC analysis. It becomes clear that the conversion levels off after 45 min for both glycopeptides. Interestingly, under the same chemoenzymatic glycosylation conditions there was a large difference in maximum conversion: while **19** reaches a conversion of ca. 80%, glycopeptide **18** stopped at only 52% conversion. The likely hydrolysis of products **18** and **19** by Endo A, thereby lowering the yield of product, was not observed within 24 h. Although at this stage no sound explanation can be provided for the difference in suitability between the two substrates, one could reason that a better isosteric reminiscence of the *N*-linked triazole to the natural amide linkage might play a role.

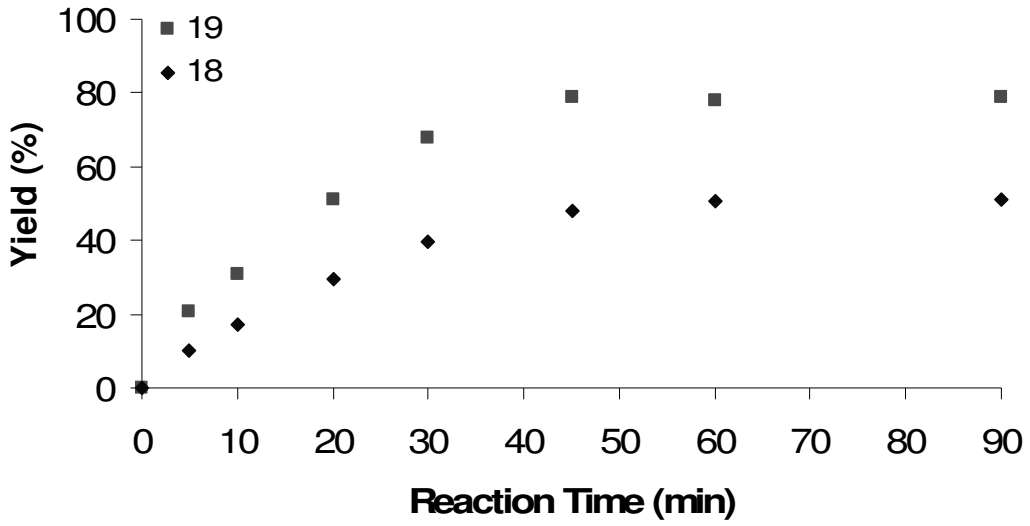
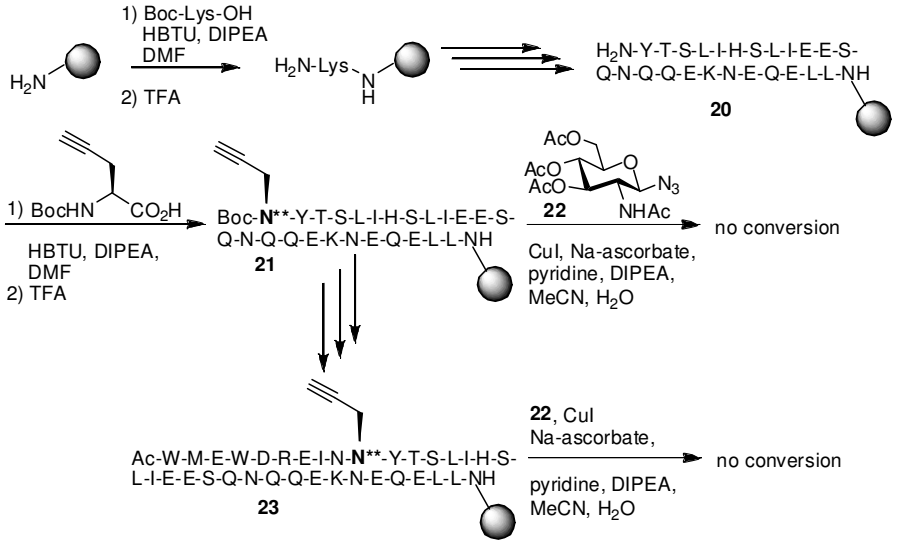


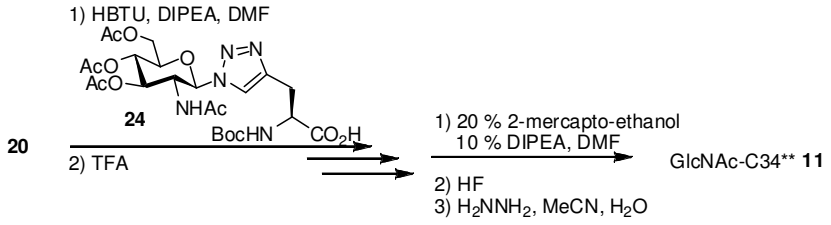
Figure 4.3 Formation of glycopeptides **18** and **19**. The oxazoline was added in 3-fold excess.

Since it was discovered that conversion of the *C*-linked glycopeptide **13** into **18** proceeded less efficiently as compared to conversion of **16** into **19**, incorporation of the carbohydrate fragment onto C34 domain via the *N*-triazole linkage (GlcNAc-C34**, **11**) was considered the more logical option.



Scheme 4.10 SPPS and CuAAC reactions on the resin

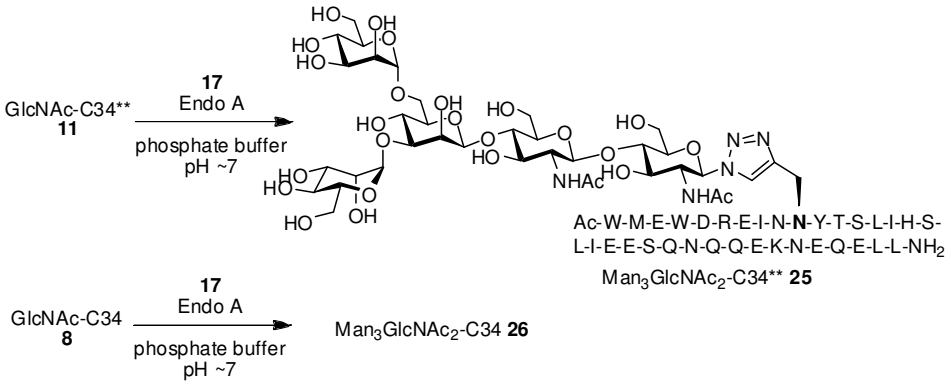
Thus, GlcNAc-C34** (**11**) was synthesized on a resin using solid phase peptide synthesis (SPPS) by using the standard Boc strategy.[21] Two principal strategies can be followed, *i.e.* incorporation of a triazole-linked glycoamino acid in the growing peptide chain or a two-step approach involving initial incorporation of propargylglycine followed by introduction of the carbohydrate by 1,3-dipolar cycloaddition. Since the latter strategy is more modular in nature, *i.e.* it allows the attachment of a variety of sugars post-solid phase synthesis, it was investigated first. Therefore, propargylglycine[20] was incorporated in the peptide synthesis sequence, followed by ligation with glucosamine **22** using the CuAAC reaction (Scheme 4.10). Unfortunately, CuAAC reaction on the resin did not succeed, neither on the terminal (**21**) nor on the internal (**23**) propargylglycine residue.



Scheme 4.11 SPPS of glycopeptide GlcNAc-C34** (**11**) with glycoamino acid **24**

In contrast, the desired glycopeptide **11** was smoothly obtained by incorporation of the glycoamino acid **24**[20] (Scheme 4.11). The protecting groups were removed from

the peptide backbone and GlcNAc *O*-6, but it was found that after cleavage from the resin two acetyl groups on *O*-3 and *O*-4 were retained requiring removal with hydrazine.[22] The thus obtained product was purified by preparative reversed-phase HPLC and the structure established as being **11** by characterization using ESI-HRMS.



Scheme 4.12 Endo A-catalyzed glycosylation of the mimic (**11**) and wild-type (**8**) GlcNAc-C34

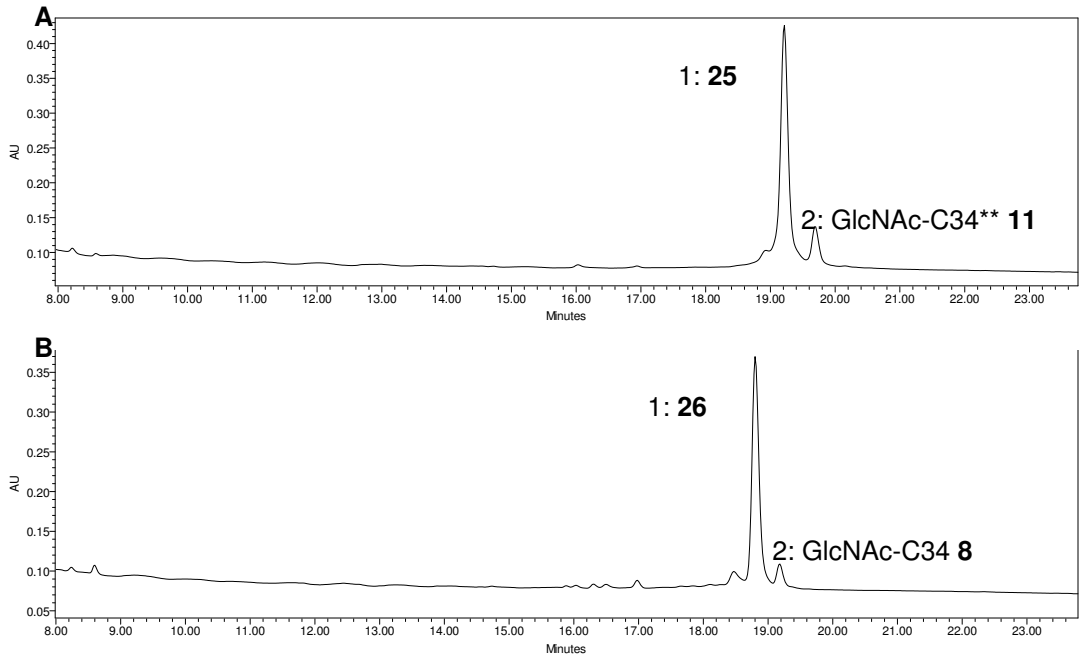


Figure 4.4 HPLC conversions of GlcNAc-C34 and GlcNAc-C34**. **A**, mimic **11** into **25** and **B**, native **8** into **26**

GlcNAc-C34** (**11**), as well as native GlcNAc-C34 (**8**),[16] were glycosylated with the sugar oxazoline donor **17** under the action of Endo A. Gratifyingly, after 2 hours full

conversion (>95%) was observed by HPLC for both glycopeptides (Figure 4.4). The product peaks from the HPLC analyses were fractionated and the structures were confirmed by ESI-MS (Figure 4.5).

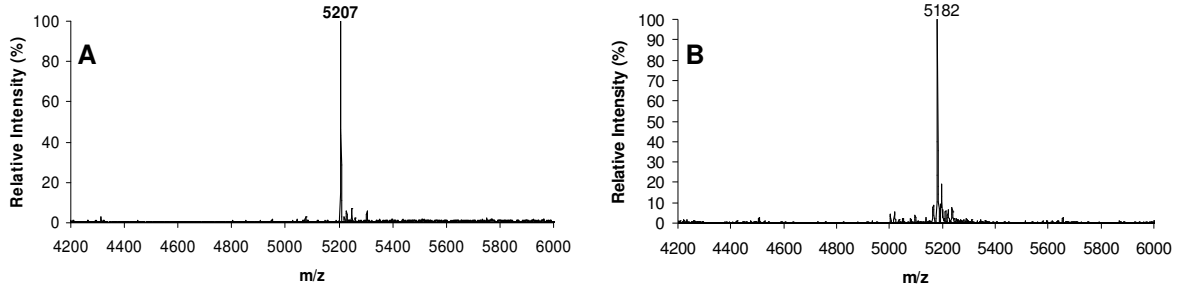
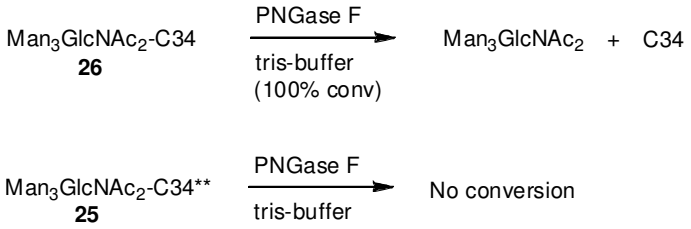


Figure 4.5 ESI-MS spectra of **A** $Man_3GlcNAc_2$-C34** (**25**) and **B** $Man_3GlcNAc_2$-C34 (**26**)

4.3 Stability towards enzymes

Peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F (PNGase F) is an example of a peptide *N*-glycanase that cleaves the β-aspartylglucosamine bond of asparagine-linked oligosaccharides, thereby converting the asparagine residue into an aspartic acid. The enzyme PNGase F has a broad substrate specificity, but both the amino and carboxyl groups of the asparagine residue have to be in a peptide linkage, while the oligosaccharide must consist at least of the *N,N'*-diacetylchitobiose core, GlcNAc-(β1→4)-GlcNAc. Therefore, subjection of either **25** or **26** to the action of PNGase F provides a good insight into the stability of the triazole-linked glycopeptide **25** towards enzymes (Scheme 4.13). Much to our satisfaction, the native glycopeptide **26** was completely hydrolyzed within 1 hour, whereas the triazole-linked glycopeptide **25**, as expected, appeared unaffected (Figure 4.6).



Scheme 4.13 PNGase F-catalyzed hydrolysis of glycopeptides **25** and **26**

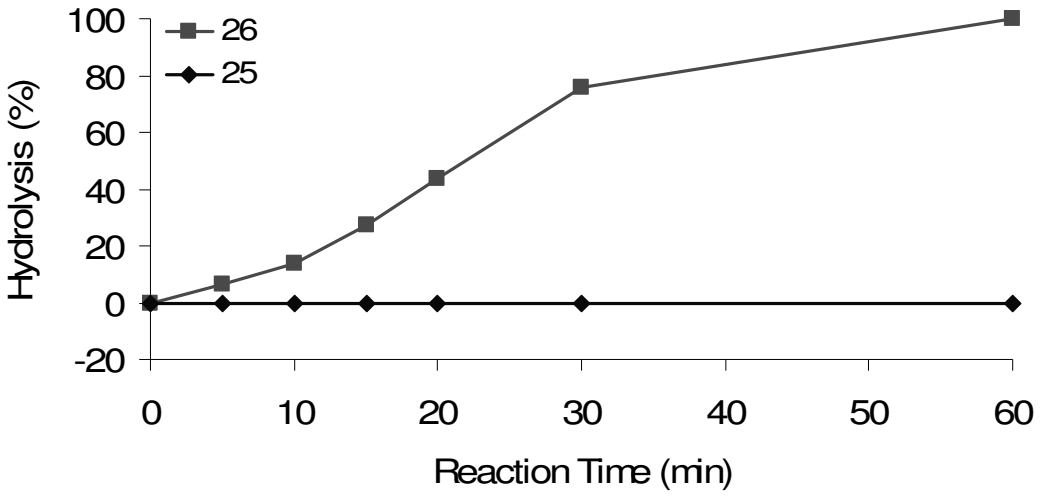


Figure 4.6 Hydrolysis of glycopeptides **25** and **26**

4.4 Conclusions

Triazole-linked glycopeptides can be effectively obtained by glycosylation of monosaccharide peptides with carbohydrate oxazolines catalyzed by Endo A, with a clear preference for the *N*-linked glycopeptide over the *C*-linked glycopeptide. The latter phenomenon may be explained by better isosteric mimicry of the *N*-triazole to the natural amide bond of *N*-glycoproteins. Apart from dipeptides, Endo A-catalyzed transglycosylation appeared also effective for a 34-mer synthetic glycopeptide, obtained by incorporation of a Boc-protected glycoamino acid in the growing peptide chain. The alternative approach, involving CuAAC reaction of an azidosugar onto the resin containing an acetylene-containing amino acid was not successful, although only preliminary results were shown. Possibly, a better efficiency can be obtained by switching to a solvent such as DMF in order to avoid shrinkage of the resin as is the case with acetonitrile.

Finally, we have shown that the triazole-linked glycopeptide showed complete resistance towards PNGase F, an encouraging finding with respect to future *in vivo* applications of triazole-linked glycoproteins for *e.g.* imaging purposes or pharmaceutical use.

4.5 Outlook

Currently, the triazole-linked glycopeptide **25** is being evaluated for its ability to form six-α-helix bundles with the corresponding *N*-terminal peptide fragment N36. Both C34 and N36 complexes will be analyzed by circular dichroism (CD), to give a measure for the α-helical content of the complex. Moreover, the measurement of the anti-HIV activity of the triazole-linked C34 glycopeptide **25** and the native glycopeptide **26** are in progress and their antiviral activities will be compared.

4.6 Acknowledgements

Dr. Brian H.M. Kuijpers is acknowledged for providing the *N*-glycoamino acid and dipeptide. Dr. Tilman Hackeng and Wenke Adriaens (Cardiovascular Research Institute Maastricht, University Maastricht, The Netherlands) are kindly acknowledged for their contribution to the solid phase peptide synthesis. We are grateful to Prof. Dr. Lai–Xi Wang and Dr. Wei Huang (Institute of Human Virology, University of Maryland, Maryland, USA) for performing the chemoenzymatic glycosylation experiments and the fruitful collaboration.

4.7 Experimental section

For general experimental details, see section 2.6. Analytical reversed phase high pressure liquid chromatography (RP-HPLC) was performed on a Varian Prostar HPLC system with a Vydac™ protein peptide C18 column (0.5 × 15 cm, flow 1 mL/min), eluted with a linear gradient of 0–60% CH_3CN in 0.1% aqueous TFA in 30 minutes. The HPLC system was coupled to a UV/Vis detector probing at 214 nm. Semipreparative RP-HPLC was performed on a HP1050 HPLC system with a Vydac™ C18 column (2.5 × 20 cm, 10 mL/min), eluted with a linear gradient of 0-60% MeCN in 0.1% aqueous TFA in 90 minutes. LRMS (ESI-MS) was performed on a SCIEX API-150 EX single quadruple mass spectrometer.

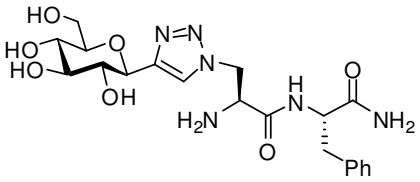
General procedure (glycosylation with sugar oxazoline)

A mixture of the $Man_3GlcNAc$-oxazoline **17** (290 nmol) and the glycopeptide (100 nmol) in a phosphate buffer (40 µL, pH 7.0, 50 mM) was incubated at 23 °C with the enzyme Endo A (0.4 µg). The reaction was monitored by analytical HPLC on a Waters Nova-Pak C18 column (3.9 × 150mm) at 40 °C with a linear gradient (0-90% MeCN containing 0.1% TFA in 30 min, flow rate 1 mL/min). The GlcNAc-peptide was converted into a new species that was eluted slightly earlier than the starting material. The chemoenzymatic reaction was stopped by heating in a boiling water

bath for 3 min. The product was purified by preparative HPLC on a Waters preparative column (Symmetry 300, 19 × 300 mm) to afford the glycopeptide.

Procedure for PNGase digestion
Glycosylated C34 peptides (**25** or **26**, 30 µg) in Tris-Cl buffer (20 µL, pH 7.5, 20 mM) was incubated at 37 °C with PNGase (6 U). The digestion was monitored by analytic HPLC under the conditions as applied in enzymatic glycosylation reactions.

L-T1M[4-(β-D-Glc)]-L-Phe-NH_2 (13)

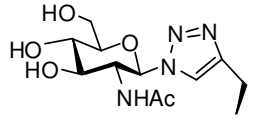


Cbz-L-T1M{4-[β-D-Glc$(Ac)_4$]}-L-Phe-NH_2 (**12**; 73 mg, 0.095 mmol) was dissolved in MeOH (1 mL), and K_2CO_3 (2 mg, 9 µmol) was added. The reaction was stirred for 1.5 h at room temperature. The crude mixture was neutralized with Amberlite IR 120 (prewashed with MeOH), filtered and evaporated to obtain the deacetylated product. This was dissolved again in MeOH (2 mL) and Pd/C (12 mg, 10 µmol) was added. The reaction was stirred overnight under an atmospheric pressure of H_2. The suspension was filtered and the solvent was evaporated. The product was lyophilized from AcOH (1 M in H_2O) to yield the desired product **13** (35 mg, 71%). ^{1}H-NMR (400 MHz, D_2O) δ = 8.04 (s, 1 H), 7.51-7.31 (m, 5 H), 4.72-4.50 (m, 4 H), 4.06-3.90 (m, 2 H), 3.84-3.74 (m, 2 H), 3.72-3.55 (m, 3 H), 3.20 (dd, *J* = 13.9, 6.5 Hz, 1 H), 3.06 (dd, *J* = 13.9, 8.4 Hz, 1 H). ^{13}C NMR (75 MHz. D_2O) δ = 174.7, 172.6, 172.6, 144.2, 135.8, 128.7, 128.2, 126.7, 125.1, 79.5, 76.5, 72.9, 72.4, 69.0, 60.3, 54.0, 52.5, 36.5. HRMS (ESI) *m/z* calculated for $C_{20}H_{29}N_6O_7$ $[M+H]^+$ 465.20977; found 465.20851.

L-T1M[4-(Man$_3$GlcNAcGlc)]-L-Phe-NH_2 (18)
Applying the general procedure to **13** gave **18** (52% conversion). ESI-MS *m/z* calculated for $C_{46}H_{73}N_7O_{27}$ $[M+H]^+$ 1155; found 1155.

L-Pro-L-T4M[4-(Man$_3$GlcNAcGlc)]-OH (19)
Applying the general procedure to **16** gave **19** (79% conversion). ESI-MS *m/z* calculated for $C_{42}H_{69}N_6O_{28}$ $[M+H]^+$ 1105; found, 1105.

GlcNAc-C34 (11)**



Ac-W-M-E-W-D-R-E-I-N-**N***-Y-T-S-L-I-H-S-L-I-E-E-S-Q-N-Q-Q-E-K-N-E-Q-E-L-L-NH_2

The peptide was obtained via manual solid phase peptide synthesis (SPPS) using the *in situ* neutralization/HBTU activation procedure for Boc chemistry on a MBHA resin.[23] *N*-terminus acetylation was performed by 2 × 2 min treatment with 1/1 *v/v* Ac_2O/pyridine (5 mL 0.5 M in DMF). DNP was removed from the His with 2 × 30 min treatment with a mixture of DIPEA (1% *v/v*) and mercaptoethanol (4% *v/v*) in DMF (7 mL). Formyl groups were removed by treatment with a continuous flow of piperidine (250 mL; 20% *v/v* in DMF) in 8 min. The Boc groups were removed with a 2 × 1 min treatment with TFA. After a DMF, DCM, and a

1:1 *v/v* MeOH/DCM flow wash, the resin was dried under vacuum. HF cleavage (4% *v/v* of *p*-cresol added as a scavenger) and subsequent lyophilization gave the acetylated crude product. The acetyl protected glycopeptide was dissolved in 10% MeCN in H_2O (1 mg/1 mL) and treated with hydrazine hydrate (10% *v/v* in H_2O) for 2 h.[22] The fully deprotected GlcNAc-C34** was purified by preparative RP HPLC over a C18 column. The product was analyzed by HRMS (ESI): calculated for $C_{195}H_{299}N_{54}O_{68}S$ $[M+3H]^{3+}$ 4517.13193; found 4517.11479 (based on the deconvolution of the data).

$Man_3GlcNAc_2$-C34 (25)**
Applying the general procedure to **11** gave **25** (100% conversion). ESI-MS calculated for $C_{221}H_{342}N_{55}O_{88}S$, M = 5207; found, 5207 (deconvolution by MaxEnt).

$Man_3GlcNAc_2$-C34 (26)
Applying the general procedure to **8** gave **26** (100% conversion rate). ESI-MS calculated for $C_{220}H_{342}N_{53}O_{89}S$ M = 5182; found, 5182 (deconvolution by MaxEnt).

4.8 References

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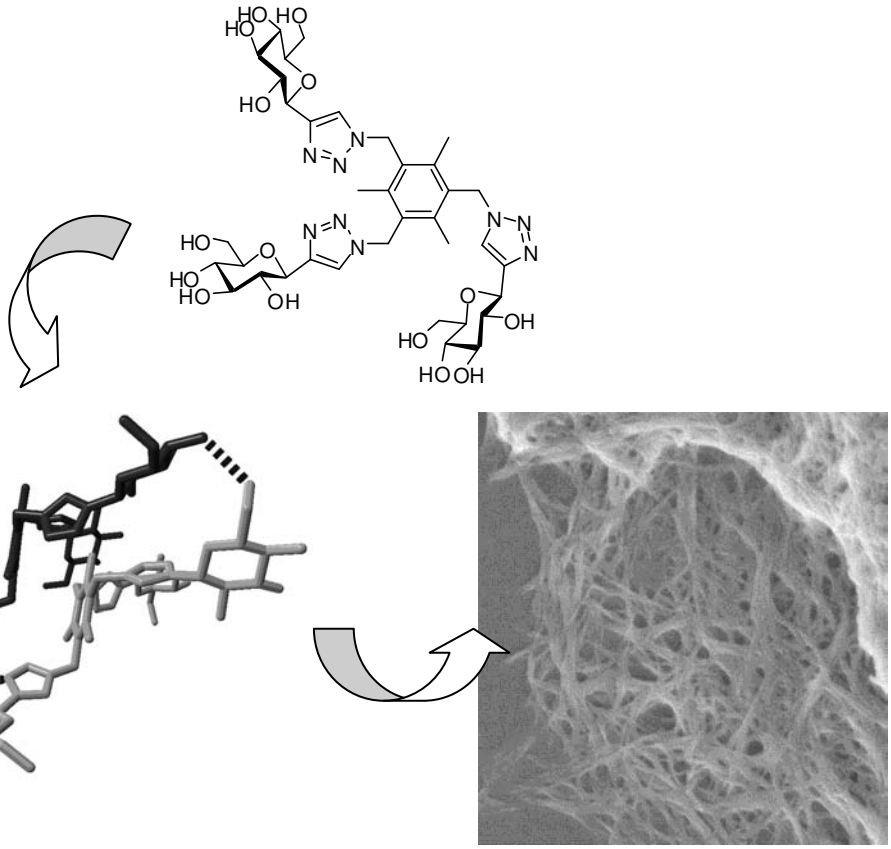
5 Supramolecular gel formation of triazole-linked oligosaccharides

Abstract

Glycoacetylenes were condensed with tri- and hexaazidohexamethylbenzene in the presence of a copper-wired stirring rod. The resulting triazole-linked oligosaccharides were found to form supramolecular gels, presumably by a combination of hydrogen bonding between the saccharide moieties and π-π stacking of the arene moieties, as indicated by molecular modeling. The trisaccharide gel was visualized using SEM, TEM and AFM techniques, all of which manifested the presence of intertwined fibers.



5.1 Formation of supramolecular gels

A gel primarily consists of a solvent (or in exceptional cases air or gas) and an immobilizing agent. Typically, a solvent is entrapped in a 3D-network of the immobilizing agent, formed through cross-linking of polymeric strands by chemical binding, physical forces or mechanical entanglements.[1-3]

There are many types of gels. One can make a classification depending on the medium or the source(Figure 5.1).[4]

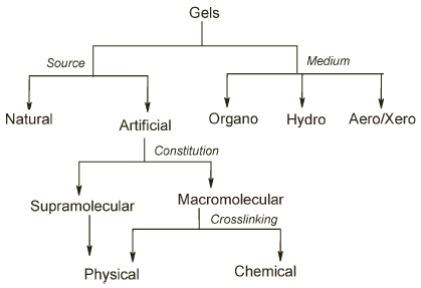


Figure 5.1 Classification of gels based on source or medium

The simplest of the two categorizations is based on the medium in which the gel is formed. In general, gels form either in an organic solvent or a protic, polar solvent. Alternatively, gelation without solvent can also be recognized.

A more sophisticated classification focuses on the source of the gelator. Thus, gels can be divided in natural and artificial gels. Artificial gels, in turn, can be subdivided based on constitution, *i.e.* supramolecular or macromolecular gels. A final subdivision of macromolecular gels, consisting of 3D-network forming polymers, reflects either physical or covalent bonding.

A supramolecular gel consists of a 3D-network of non-covalently bound (*e.g.* via hydrogen bonding) fibers, which themselves are also the result of non-covalent interactions (Figure 5.2). The self-assembly of such small molecule gelators into Self-Assembled Fibrillar Networks (SAFINs) is accomplished via hydrogen bonds, ionic bonds, van der Waals interactions, hydrophobic or hydrophilic interactions, metal coordination or π-π stacking.[2] Because a supramolecular gel is built up entirely from non-covalent interactions, gel formation is reversible and the gel can be transformed

into a fluid by external factors such as temperature, pressure, concentration or addition of other solvents.

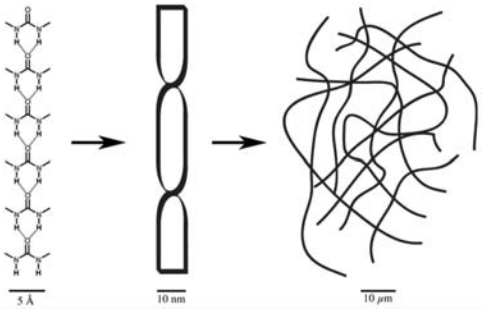


Figure 5.2 Self-assembly of a supramolecular gel[5]

A supramolecular gel is normally formed by heating a solution of a gelator and cooling the supersaturated solution to room temperature. Different possibilities arise when the solution is cooled (Figure 5.3). A gel is obtained when the gelator does not precipitate or form a crystal (as the result of multidirectional aggregation), but self-assembles instead into a cross-linked 3D-network.[2, 3, 6]

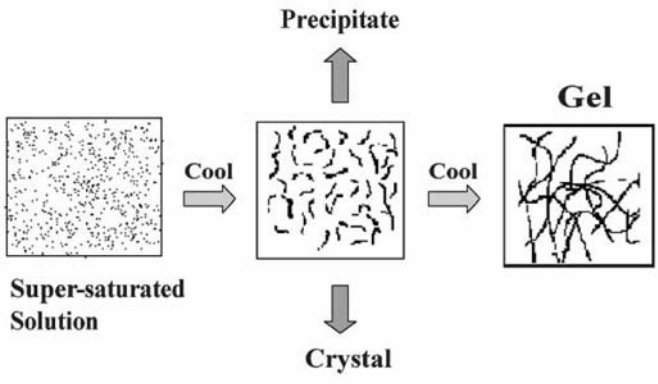


Figure 5.3 Schematic representation of gel aggregation

The structure of a supramolecular gel and the conditions to obtain a gel from a gelator solution are currently understood at the supramolecular level. However, much less is known about the structure of the building blocks of the gel, *i.e.* the gelators. Van Esch *et al.* has proposed guidelines of properties that gelators should possess, which are: 1) complementary and unidirectional intermolecular interactions to favor fibrillar formation; 2) sufficiently high solubility to prevent crystallization; and 3) potential to form fiber cross-links for 3D-network formation.[7]

Taking these prerequisites in consideration, it becomes possible to design a molecule destined to form a gel. For example, unprotected glycosides have a high solubility and are able to form intermolecular hydrogen bonds that can aid in the formation of a cross-linked 3D-network. According to the guidelines, the glycoside should also have unidirectional intermolecular interactions. We envisioned that an arene core provides a suitable scaffold for the formation of a unidirectional fiber via π-π stacking (Figure 5.4). Based on the glycoacetylenes (Chapter 3), a logical retrosynthetic disconnection from the substituted benzene ring leads to an arene core charged with azides, thereby allowing access to the projected gelator via the well-established CuAAC reaction (Figure 5.4).

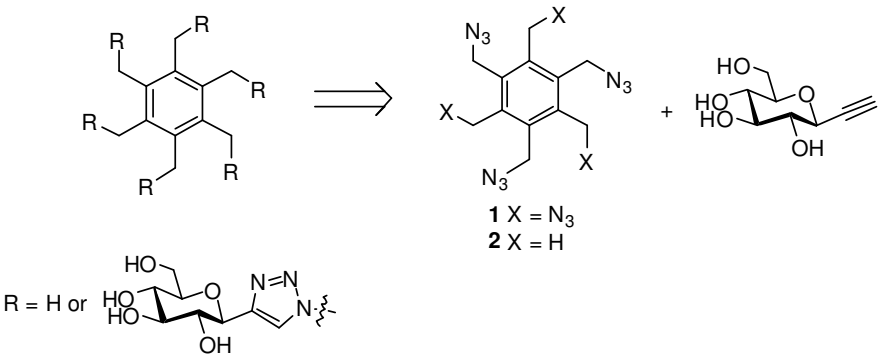
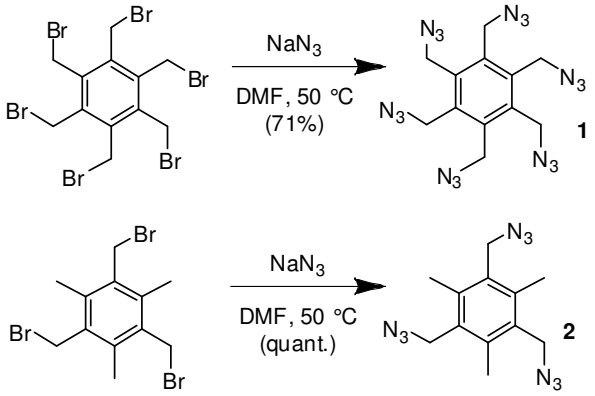


Figure 5.4 Retrosynthesis of trimeric or hexameric glycoside

5.2 Synthesis

In order to equip the benzene scaffold with hexa- and trisaccharides, the requisite hexaazidohexamethylbenzene (HAB, **1**) and triazidohexamethylbenzene (TAB, **2**) were prepared first from the corresponding bromides (Scheme 5.1). [8, 9]



Scheme 5.1 Synthesis of tri- and hexaazidohexamethylbenzene **1** and **2**, respectively

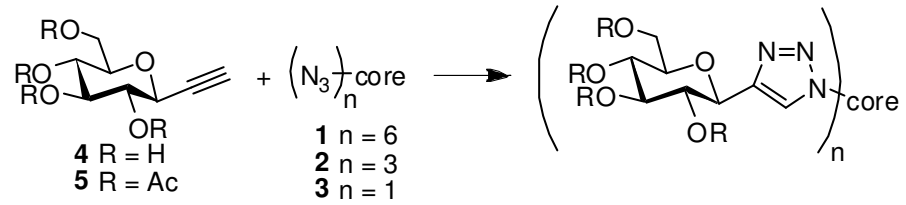
Since Gilbert *et al.*[8] synthesized HAB as a potential explosive, precaution was compulsory. Indeed, DSC measurements of **1** showed exothermal decomposition energy of 3621 kJ/kg when heated above the melting point (166 °C). Although this high temperature suggests that **1** can be handled relatively safely at ambient temperature, it is uncertain if unknown material (*e.g.* metal ions) can initiate undesired decomposition.

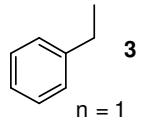
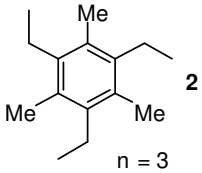
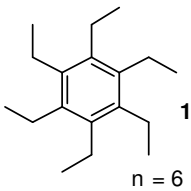
Initially, cycloaddition experiments were conducted with three azido compounds, *i.e.* benzyl azide, HAB (**1**) and TAB (**2**). As the acetylenic coupling partner, both unprotected and acetyl-protected glycoacetylenes **4** and **5** were employed.[10] Recently, we established[11] that CuAAC of acetylenic sugars and azido-modified amino acids is best performed under the action of $Cu(OAc)_2$ and sodium ascorbate in a H_2O/*t*-BuOH mixture. Thus, under the same conditions, 69% yield was obtained for cycloaddition of benzyl azide (**3**) with fully unprotected glycoacetylene **4** (Table 5.1, entry 1). Since it was realized that a higher yield was desirable for the multiply substituted benzene derivatives, cycloaddition was also attempted under the action of a copper-wired stirring rod, leading to full conversion of starting materials and a quantitative yield of the desired triazole adduct (entry 2). On the other hand, no difference in yield was observed between the different conditions upon CuAAC reaction of benzyl azide with the acetyl-protected sugar.

A completely different situation occurred in case of the triple CuAAC addition of compound **4** or **5** onto triazide **2**. The unprotected sugar gave little or no incorporation at all (entries 5 and 6) irrespective of the conditions employed. On the

other hand, the acetyl-protected sugar smoothly reacted with the triazide under either conditions, leading to quantitative (entry 7) or excellent (entry 8) yield of the desired adduct. It must be noted that a slightly elevated temperature was employed for the copper-wire-catalyzed reaction. Finally, the conditions with $Cu(OAc)_2$ or copper-wire were tried in the CuAAC reaction with HAB (**1**). Unfortunately, for this particular azide no complete glycosylation took place for the unprotected carbohydrate (entries 9 and 10). More surprisingly, a striking difference in coupling efficiency was observed for the acetylated sugar **5** depending on the source of copper (entry 11 and 12).[12]

Table 5.1 CuAAC reactions of glycoacetylenes **4** and **5** with azido-substituted arenes

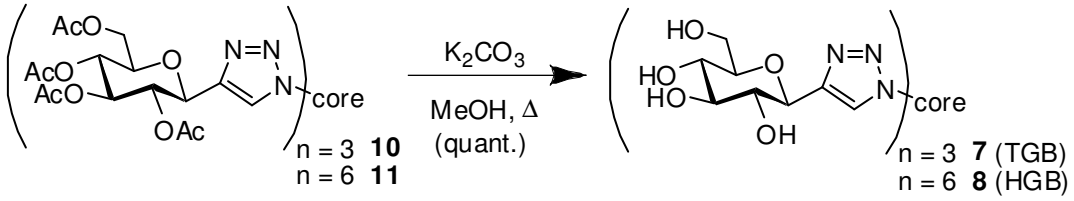


entry	core	R	conditions [a]	yield	product
1		H	A	69%	**6**
2			B	quant.	**6**
3		Ac	A	quant.	**9**
4			B	quant.	**9**
5		H	A	6%	**7**
6			B	- [b]	**7**
7		Ac	A	quant.	**10**
8			B	84%	**10**
9		H	A	- [b]	**8**
10			B	- [b]	**8**
11		Ac	A	- [b]	**11**
12			B	87%	**11**

[a] Conditions: A. $Cu(OAc)_2$, Na-ascorbate, H_2O/*t*-BuOH (1:1); B. copper-wire, MeCN, 40 °C; [b] Multiple partially glycosylated products.

The acetyl-groups of tri- and hexameric glucosides **10** and **11** were removed under the action of K_2CO_3 in methanol (reflux, 1 h) to form 1,3,5-*tris*-

(glucosyltriazolylmethyl)-2,4,6-methylbenzene (TGB, **7**) and 1,2,3,4,5,6-*hexakis*-(glucosyltriazolylmethyl)benzene (HGB, **8**) as depicted in Scheme 5.2.



Scheme 5.2 Deprotection of tri- and hexameric glucosides **10** and **11**

5.3 Supramolecular behavior of TGB and HGB

5.3.1 Modeling

In order to estimate the likeliness of supramolecular aggregation, information was needed about the molecular conformation of TGB and HGB first. To this end, some attempts were undertaken to obtain crystals for X-ray diffraction,[3] but unfortunately no suitable crystals could be grown. Alternatively, semi-empirical modeling studies could give an indication about the conformational preference of the sugars.

As shown in Figure 5.5, HGB can exist in eight specific conformers, depending on the glucotriazolyl moiety pointing up (A) or down (B) with respect to the plane of benzene core. The situation for TGB is much less complex, since only two possible conformers can exist, either with all substituents at the same side of the plane or an AAB-type conformation.

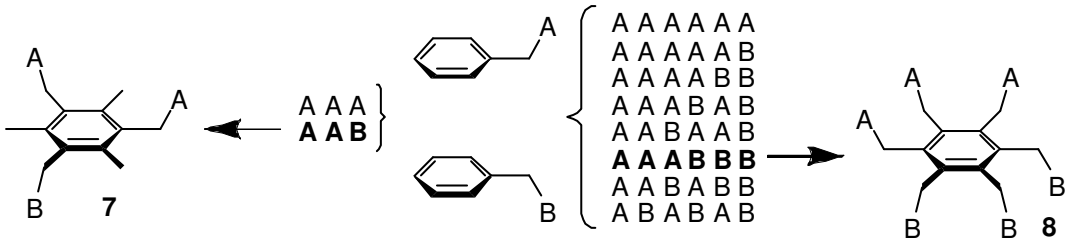


Figure 5.5 Possible TGB and HGB conformers

The structures were calculated using Spartan '04 from Wavefunction, Inc, Irvine, CA and obtained by a geometry optimization in vacuum, using the semi-empirical module with PM3 parameters. PM3 is a semi-empirical method for the quantum

calculations of molecular electronic structure that was optimized for organic molecules.[13, 14]

For steric reasons, one may assume that the AAB conformer of TGB is the lowest energy conformer. Surprisingly, PM3 calculations established the AAA conformation of TGB as the more stable conformer (AAB is 5.9 kcal/mol higher in energy). Presumably, intramolecular hydrogen bonding between the carbohydrate moieties is the reason for the preference for the AAA conformation. It has to be mentioned that the suggested preference for AAA conformation is based on calculations in vacuum and that factors like solvation might give different results. From the calculated geometry, the dimensions of the molecule could be readily determined (Figure 5.6).

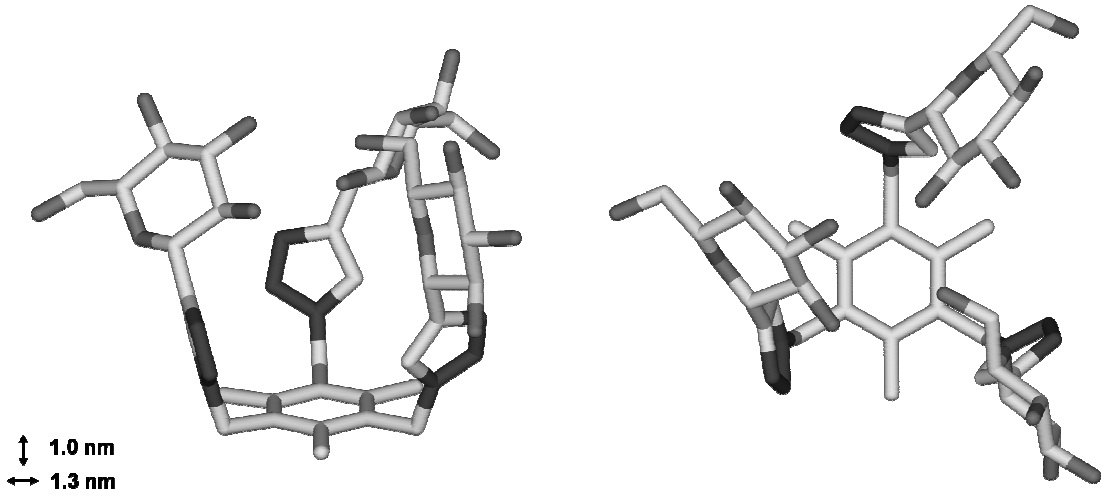


Figure 5.6 3D image of the calculated AAA conformation of TGB

For HGB, PM3 calculations predict the lowest energy for the expected ABABAB conformation (Figure 5.7). Also in this case, the dimensions of the molecule HGB were determined from the calculated geometry (Figure 5.8).

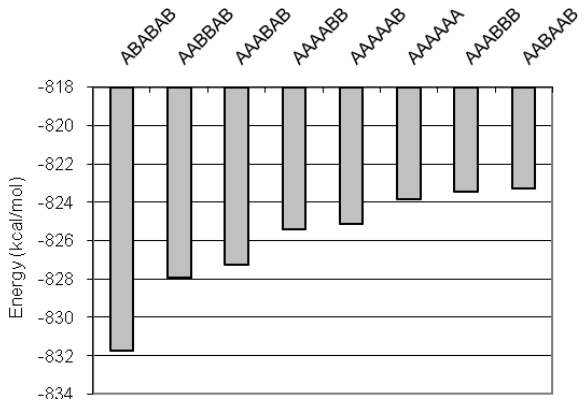


Figure 5.7 Calculated relative energies of the eight conformers of HGB

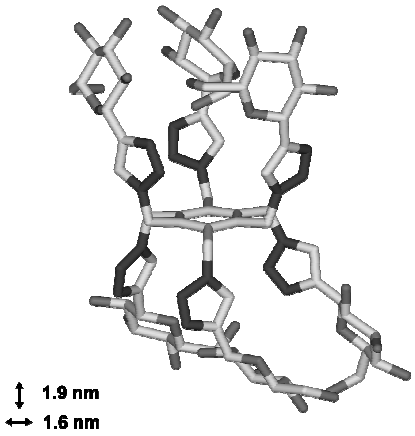


Figure 5.8 3D image and dimensions of the ABABAB conformation of HGB

It is clear, however, that the optimal geometry of a single molecule in vacuum does not necessarily reflect the conformation of the same substrate during gel-formation. Therefore, PM3 geometry optimization was also performed on the stacked form of two molecules of TGB.* It became clear that now the AAB conformer was more favored and stacked nicely in the direction perpendicular to the benzene core (Figure 5.9). It is not completely sure if the π-π stacking assures the linear nature of the strand

* Unfortunately, the stacking of HGB could not be measured.

since the distance in this between the benzene-cores is approximately 4.8 Å which is significant larger than the ideal distance of 3.4 Å. The hydroxyl groups add additional stability through intermolecular hydrogen bonding. The diameter of this supramolecular strand is 1.8 nm.

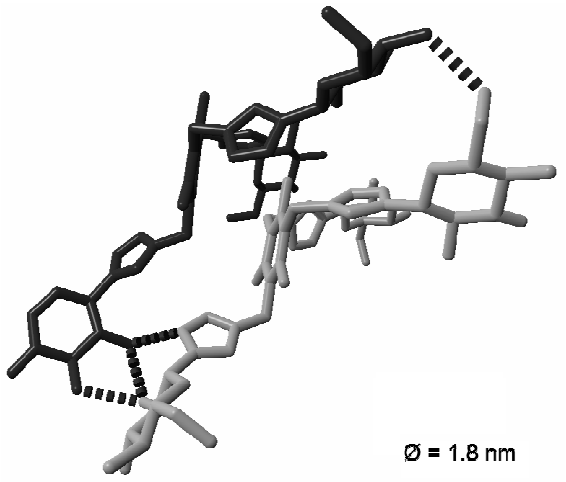


Figure 5.9 3D image for the calculated conformation for two stacked TGB molecules

5.3.2 Gel formation

Normally, gels are formed through a process that closely resembles recrystallization:[1] a supersaturated solution is cooled, resulting in the formation of a gel. The possibility of gel formation was tested for both TGB and HGB in the only solvents that could be used to dissolve the substrates, *i.e.* H_2O, DMSO and DMF. Unfortunately, no gel formation was observed in any of these solvents for either oligomeric glycoside at different concentrations up to saturation. Furthermore addition of variety of anti-solvents (like toluene, MeOH, EtOH, CH_2Cl_2, MeCN, EtOAc, THF, and dioxane) to solutions of TGB and HGB in H_2O, DMSO and DMF led to precipitation in stead of the desired gelation.

A higher concentration of TGB and HGB in these solvents (H_2O, DMSO and DMF) was expected to enlarge the chance of successful gel formation. Therefore, supersaturated solutions of TGB (8.5% w/w) and HGB (5.0% w/w) were prepared in H_2O, DMSO or DMF by sonication. Unexpectedly, no clear solutions were obtained but to our satisfaction, gel formation occurred for both oligomeric glycosides in DMF. Apparently, the added energy of the sonication process was necessary to break intramolecular hydrogen bonds and molecule–solvent interactions, in order to form

intermolecular hydrogen bonds of the gel. These intermolecular hydrogen bonds, combined with the linear character of the presumed π-π stacking interactions, led to a supramolecular strand.

Surprisingly, gel formation still occurred when the concentrations of TGB and HGB in DMF were decreased. The critical gel concentration (C_{gel}), the minimal concentration at which a gel could be formed, was determined for both oligomeric glucosides: C_{gel} of HGB is 4.6% w/w and the C_{gel} of TGB is 8.1% w/w. Typical concentrations reported are below 15%,[15] although very low concentrations have been reported in the case of sugar-derived "supergelators".[16] The nearly double value C_{gel} of TGB with respect to the C_{gel} of HGB can be explained by the fact that HGB contains more glycosides compared to TGB. Presumably, HGB can therefore form more hydrogen bonds than TGB at the same concentration.

The gelation temperature (T_{gel}), the temperature at which the gel dissolves again, was also determined for the oligomeric glucoside: T_{gel} of TGB (8.3% w/w) is 96 °C. The T_{gel} of HGB (5.9% w/w) could not be determined exactly since DMF has a boiling point of 153 °C and at this temperature the gel did not yet dissolve. Moreover this indicates that the HGB gel consist of strong interactions.

5.3.3 Scanning Electron Microscopy

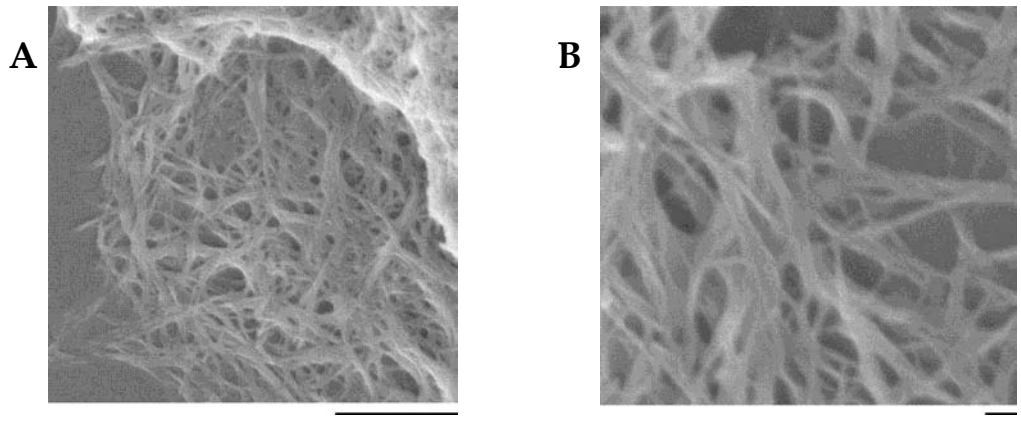


Figure 5.10 SEM images of the TGB-gel in DMF; A) bar is 1 μm; B) bar is 200 nm

The TGB-gels described above were studied with a range of microscopic techniques (*i.e.* SEM, TEM, AFM) to visualize the gel structures.† Several scanning electron microscopy (SEM) images were obtained from the TGB-gel (Figure 5.10). The 3D-network, distinctive for a gel consisting of intertwined fibers,[17] was clearly visible. The various fibers did not have a consistent thickness, which can be taken as an indication that there was not a single way in which the strands of one molecule interact to form larger fibers.

5.3.4 Transmission Electron Microscopy

After determination of the gel morphology with SEM, the dimensions of single strands were also determined with transmission electron microscopy (TEM).

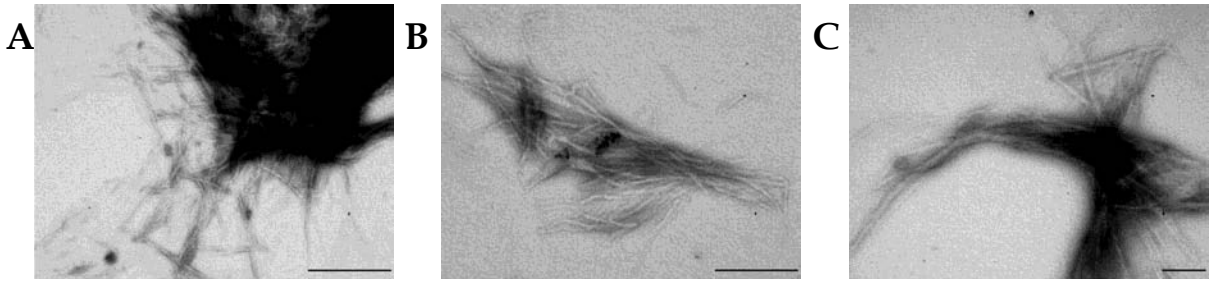


Figure 5.11 TEM images of TGB-gel in DMF; A) bar is 1 μm; B) bar is 500 nm; C) bar is 200 nm

The TEM images obtained from our experiments showed agglomerates of fibers, with some individual fibers distinguishable from the agglomerates (Figure 5.11).

TEM is suitable for determining the width of the observed fibers. Several individual TGB-fibers have been measured. This resulted in a distribution of fiber width over 6 to 18 nm (Figure 5.12).

† Unfortunately, the HGB-gel could not be visualized by SEM, TEM or AFM, since during sample preparation the HGB gel dissolved upon dilution with water

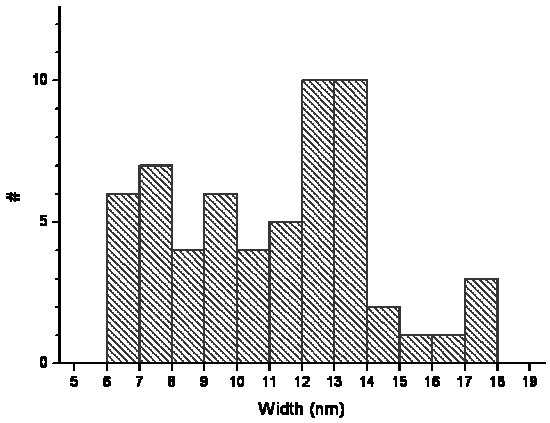


Figure 5.12 Single strand width distribution of the TGB-gel

5.3.5 Atomic Force Microscopy

Finally, the TGB-gel was studied with atomic force microscopy (AFM). The images obtained display some agglomerates as well as some individual fibers (Figure 5.13).

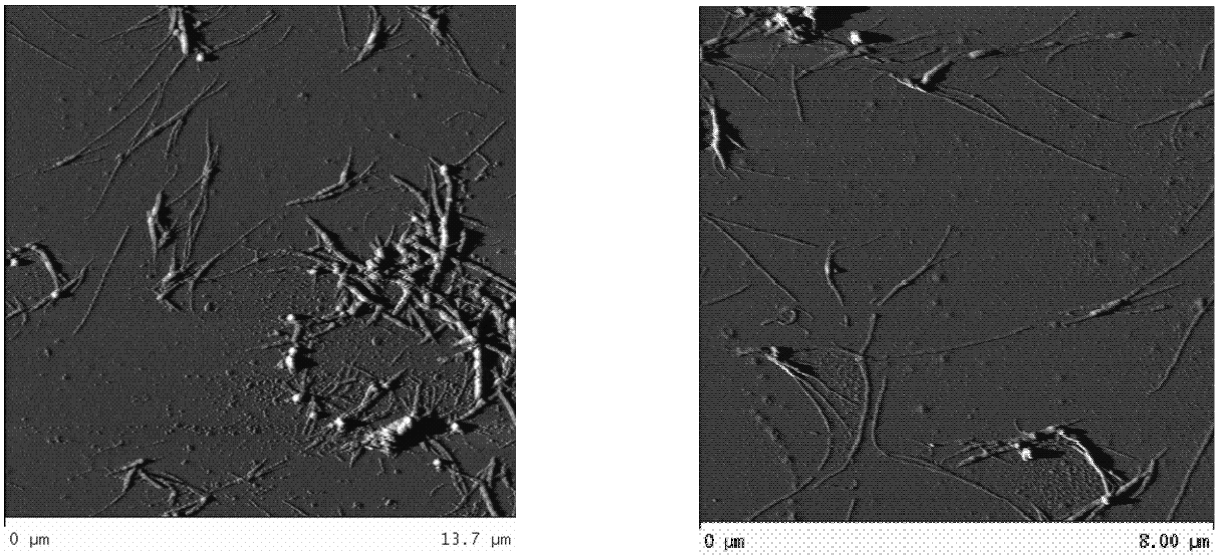


Figure 5.13 AFM images of TGB-gel in DMF

The heights of several of these fibers were determined, resulting in a height distribution as shown in Figure 5.14. The fibers that are around 8 nm in height consist possibly of two stacked fibers, which is in agreement with the majority of the measured heights of around 4 nm.

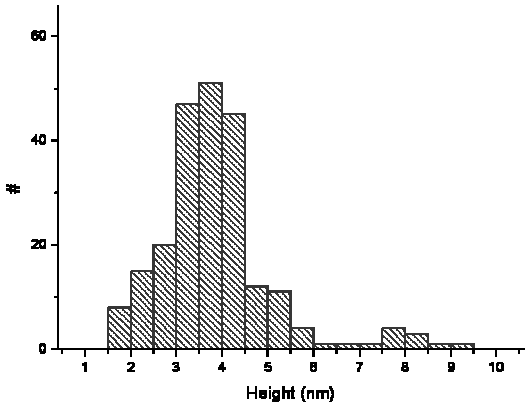


Figure 5.14 Supramolecular fiber height distribution of the TGB-gel

5.4 Gel formation hypothesis

The SEM images show a characteristic 3D-network of the TGB-gel. The AFM images visualized some individual fibers, thereby allowing height measurements. A fiber height distribution around 4 nm was clearly observed, along with another distribution around 8 nm, probably the result of overlaying fibers. In contrast, width determination based on the individual fibers visualized by the TEM images resulted in a distribution of fiber width between 6 and 18 nm. The apparent discrepancy between the different measurements based on TEM or AFM may be explained by the fact that TEM (and SEM) is measured in vacuum and AFM in ambient atmosphere. Also the nature of the sample grids may play a role: the AFM samples were prepared on hydrophobic carbon grids, whereas the TEM samples were prepared on hydrophilic Formvar grids. The fibers in the TEM sample can be wider, because the glucoside groups are drawn towards the Formvar grid through hydrophilic interactions. Moreover, the resolution of the TEM images was not as good as the resolution of the AFM images and there was not a distinct pattern in the fiber width. Therefore, it was difficult to draw a clear-cut conclusion from the fiber width data.

The diameter of a single molecular strand of TGB is 1.8 nm, according to PM3 geometry optimization. This indicates that a single fiber must consist of more than one strand. A fiber consisting of more than one strand has more intermolecular hydrogen bonds, which is beneficial to the fiber stability. The single fibers are then

intertwined to aggregate and form bigger fibers. Finally, the bigger fibers get entangled, resulting in the typical gel 3D-network.

Another possibility might be that during the sample preparation with water, the fibers absorb water, which can position between the sugar units, and cause chain swelling. Since TEM and SEM measurements are in vacuum and AFM is ambient, it might be possibly that the fibers shrink in vacuum and cling together, and therefore the width distribution is not unambiguous. This can be further investigated by AFM studies and changing the relative humidity, like Samorí *et al.* did for polyisocyanides.[18]

5.5 Conclusions

A tri- and hexaglycosylated hexamethylbenzene derivative were efficiently synthesized by the CuAAC reaction and in good yield, starting from the oligoazides and glucoacetylene **5**. The unprotected TGB (**7**) and HGB (**8**) were both able to form a supramolecular gel in DMF, using sonication at 90 °C. The TGB gel could be visualized using SEM, TEM, and AFM. Unfortunately, the HGB-gel could not be visualized by SEM, TEM or AFM, probably as a result of the dilution in water for the sample preparation procedure.

Modeling studies using PM3 calculations showed large energy differences for the postulated TGB and HGB conformers. The lowest energy conformation of TGB was AAA. However, AAB was the lowest energy conformation in the two-molecular stack calculations, due to intermolecular interactions. The lowest energy configuration of HGB was ABABAB.

5.6 Outlook

Since the work described in this chapter is a first attempt to study the behavior of these tri- and hexameric glucosides, the conclusions drawn from the measurements are not unambiguous. In order to extend the research and validate the hypotheses, several additional measurements need to be performed.

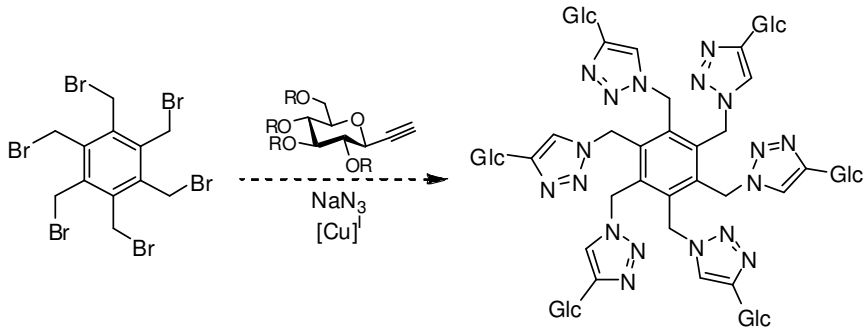
First of all, the modeling experiments need to be expanded to involve several aspects like modeling on solvated molecules instead of vacuum. The solvent can play an important role in the conformation and stacking, since the molecules can form,

besides inter- and intramolecular hydrogen bonds, hydrogen bonds with the solvent. Also, further calculations can be made on expanding the TGB stack from two to three, and stacking of two or more HGB molecules. Finally, it would be interesting to obtain an X-ray diffraction pattern from single crystals of one of the oligosaccharides. From this data, hard evidence can be obtained about the conformation of the carbohydrate moieties as well as the stacking of the molecules.

Secondly, NMR experiments from the gel in D_2O with stepwise dilution can shed light on the intermolecular interaction of the glycoclusters before gelation takes place. Chemical shifts (in particular the benzylic and triazole protons) can reveal new aspects of the self-association just before gelation, because these are characteristic for the geometry of the molecules in the final aggregates.

Furthermore, a different sample preparation should allow visualization of the HGB-gels with different microscopic techniques.

From a safety point of view, it had to be studied whether the synthesis and isolation of the oligoazides could be bypassed, for instance via a one-pot synthesis of the oligosaccharides from *in-situ* generated azides (Scheme 5.3).[17]



Scheme 5.3 One-pot synthesis of HGB via an *in-situ* generated oligoazide

5.7 Acknowledgements

Jeroen ten Dam is gratefully acknowledged for his contribution to this chapter. Ton Dirks, Matthijs Otten, Richard van Hameren, and dr. Hans Elemans are kindly acknowledged for their help with the supramolecular behavior studies and writing of this chapter. DSM is kindly acknowledged for the DSC measurement of HAB.

5.8 Experimental section

Materials

For general experimental details, see section 2.6. Peaks were assigned based on 2D analyses (gCOSY, gHSQC, and/or gHMBC). Size exclusion chromatography was performed with sephadex superdex 75 silica gel using the indicated solvent (mixture). TEM experiments were performed using a JEOL 100 CX Transmission microscope (100kV). SEM experiments were performed using a JEOL JSM T300 Scanning microscope (30kV). AFM experiments were performed using a Nanoscope III instrument from Digital Instruments.

For the semi-empirical modeling studies, the structures are calculated using Spartan '04 from Wavefunction, Inc, Irvine, CA and are obtained by a geometry optimization in vacuum, using the semi-empirical module with PM3 parameters.

Sample preparations

Gel formation: TGB or HGB was dissolved in DMF in a sealed HPLC vial. This was sonicated at 90 °C for 90 minutes.

SEM sample preparation: The TGB DMF gel was diluted with a small amount of water. A carbon grid was doped into the water and subsequently dried under vacuum. Finally it was stained with gold/platinum.

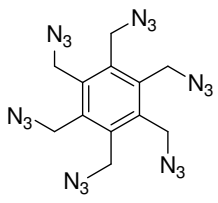
TEM sample preparation: The TGB DMF gel was diluted with water. A Formvar grid was doped into the water and subsequently dried under vacuum. There was no additional staining.

AFM sample preparation: The TGB DMF gel was diluted with water. A carbon grid was doped into the water and subsequently dried under vacuum.

Warning

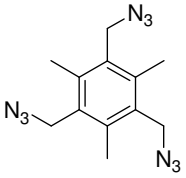
Azide containing molecules can be very explosive. Especially if more than one of this functional group is present in a relative small molecule. Nitrogen gas is released when an azide group degrades. This can result in a rapid expansion of volume, which can break the container that holds the azide compound. Hexakis-azide ***1*** *has even been tested for its explosive characteristics.*[8] *Special attention with regard to security should be paid while synthesizing and handling these azides.*

HAB (1)



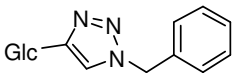
To a solution of 1,2,3,4,5,6-hexakis(bromomethyl)benzene (1.84 g, 2.89 mmol) in DMF (7 mL) was added sodium azide (1.69 g, 26.0 mmol). The mixture was stirred at room temperature for 24 h and then heated to 50 °C for 23 h. The solvent was evaporated in high vacuum and the white solid was dissolved in EtOAc. The organic phase was washed with H_2O (4 ×) and brine and dried over $MgSO_4$. Then the organic phase was concentrated *in vacuo* and recrystallization yielded **1** (0.840 g, 2.06 mmol, 71%) as a white solid. ^{1}H-NMR (300 MHz, acetone-d6) δ = 2.76 (s, 12H). IR (film) ν = 2358, 2098, 1247 cm^{-1}. Elemental Analysis: %C = 35.52, %H = 4.02, %N = 61.17. In agreement with literature.[8]

TAB (2)



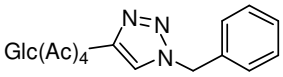
To a solution of 1,3,5-tris(bromomethyl)-2,4,6-trimethylbenzene (0.500 g, 1.26 mmol) in acetone (10 mL) was added sodium azide (0.370 g, 5.64 mmol). The mixture was stirred and heated to reflux for 24 h. The mixture was concentrated *in vacuo*. The excess of sodium azide was washed away with H_2O and **2** (0.358 g, 1.26 mmol, quant.) was obtained. R_f (EtOAc/heptane 1:10) = 0.27. ^{1}H-NMR (200 MHz, $CDCl_3$) δ = 4.50 (s, 6 H), 2.46 (s, 9 H). ^{13}C-NMR (50 MHz, $CDCl_3$) δ = 138.3, 131.0, 49.1, 16.6. IR (film) ν = 2085, 1334, 1235 cm^{-1}. Elemental Analysis: %C = 50.39, %H = 5.30, %N = 44.28. In agreement with literature.[19]

MGB (6)



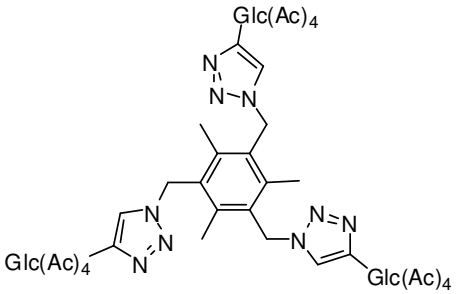
A solution of benzyl azide (**3**, 30 mg, 0.23 mmol) and **4** (27 mg, 0.14 mmol) in MeCN (2.5 mL) was stirred with a stirring bar winded with copper wire at room temperature for 7 d. The solvent and excess benzyl azide were evaporated *in vacuo* and yielded **6** (46 mg, 0.14 mmol, quant.) as a white solid. R_f (MeOH/toluene 1:5) = 0.21. ^{1}H-NMR (400 MHz, CD_3OD) δ = 7.98 (s, 1 H), 7.36-7.32 (m, 5 H), 5.58 (s, 2 H), 4.39 (d, *J* = 9.2 Hz, 1 H), 3.85 (d, *J* = 12.0 Hz, 1 H), 3.66 (dd, *J* = 4.6, 12.1 Hz, 1 H), 3.55 (t, *J* = 8.2 Hz, 1 H), 3.47-3.40 (m, 3 H). ^{13}C-NMR (75 MHz, CD_3OD) δ = 147.8, 136.7, 130.0, 129.6, 129.3, 124.8, 82.2, 79.6, 75.7, 75.0, 71.6, 63.0, 55.0. IR (film) ν = 3342, 2357, 1643 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{15}H_{19}N_3Na_1O_5$ $(M+Na)^+$ 344.12224, found 344.12199.

(Ac)MGB (9)



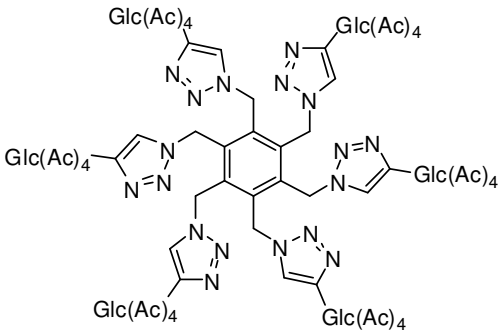
A solution of benzyl azide (**3**, 75 mg, 0.56 mmol) and **5** (0.100 g, 0.281 mmol) in MeCN (5 mL) was stirred with a stirring bar winded with copper wire at 40 °C for 49 h. H_2O was added and the product was extracted with CH_2Cl_2. The organic phase was concentrated *in vacuo* and the product was purified by chromatography (EtOAc/heptane 1:1) to obtain **9** (0.13 g, 0.27 mmol, 96%) as a white solid. R_f (EtOAc/heptane 1:1) = 0.21. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.54 (br s, Tr*H*), 7.40-7.21 (m, Ph*H*), 5.50 (s, C*H*$_2$Ph), 5.32 (t, *J* = 9.3 Hz, *H*-C(3)), 5.23 (t, *J* = 9.4 Hz, *H*-C(2)), 5.14 (t, *J* = 9.6 Hz, *H*-C(4)), 4.77 (d, *J* = 9.3 Hz, *H*-C(1)), 4.25 (dd, *J* = 4.6, 12.4 Hz, *H*-C(6)), 4.10 (d, *J* = 12.2 Hz, *H*-C(6)), 3.85 (dd, *J* = 3.3, 9.8 Hz, *H*-C(5)), 2.04 (s, OAc), 2.03 (s, OAc), 1.99 (s, OAc), 1.85 (s, OAc). IR (film) ν = 2358, 1748, 1217 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{23}H_{28}N_3O_9$ $(M+H)^+$ 490.18255, found 490.18173.

(Ac)TGB (10)



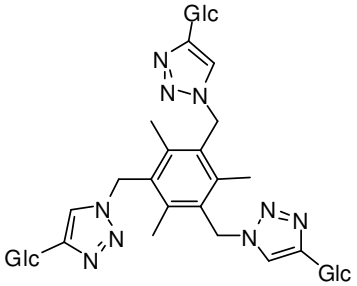
A solution of **2** (15 mg, 0.053 mmol) and **5** (62 mg, 0.17 mmol) in MeCN (2 mL) was stirred using a stirring bar winded with copper wire at 40 °C for 23 h. H_2O was added and the product was extracted with CH_2Cl_2. The organic phase was concentrated *in vacuo* and the product was purified by chromatography (EtOAc) to obtain **10** (61 mg, 0.045 mmol, 84%) as a white solid. R_f ($MeOH/CH_2Cl_2$ 1:19) = 0.10. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.42 (br s, (C=C*H*)$_3$), 5.61 (br s, (C*H*$_2$Ph)$_3$), 5.30 (t, *J* = 9.4 Hz, *H*-C(3)$_3$), 5.20 (t, *J* = 9.7 Hz, *H*-C(2)$_3$), 5.10 (dd, *J* = 9.4, 10.0 Hz, *H*-C(4)$_3$), 4.71 (d, J = 10.0 Hz, *H*-C(1)$_3$), 4.25 (dd, 5.1, 12.4 Hz, *H*-C(6)$_3$), 4.12-4.08 (m, *H*-C(6)$_3$), 3.83 (ddd, *J* = 2.3, 5.0, 10.1 Hz, *H*-C(5)$_3$), 2.33 (s, (C*H*$_3$Ph)$_3$), 2.03 (s, (OAc)$_3$), 2.02 (s, (OAc)$_3$), 1.99 (s, (OAc)$_3$), 1.84 (s, (OAc)$_3$). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.6, 170.1, 169.5, 144.2, 140.0, 130.3, 122.0, 76.3, 73.9, 73.2, 71.2, 68.5, 62.2, 49.2, 20.8, 20.6, 16.5. IR (film) ν = 2920, 2358, 1748, 1364, 1225, 1044, 603 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{60}H_{76}N_9O_{27}$ $(M+H)^+$ 1354.48506, found 1354.47769.

(Ac)HGB (11)



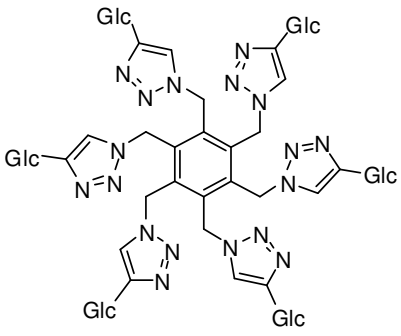
A solution of **1** (35 mg, 0.085 mmol) and **5** (0.200 g, 0.561 mmol) in MeCN (4 mL) was stirred with a stirring bar winded with copper wire at 40 °C for 90 h. H_2O was added and the product was extracted with CH_2Cl_2. The organic phase was concentrated *in vacuo* and the product was purified by gradient chromatography (100% CH_2Cl_2 to 90% CH_2Cl_2/MeOH) to obtain **11** (0.19 g, 0.074 mmol, 88%) as a white solid. R_f (CH_2Cl_2/MeOH 9:1) = 0.25. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.96 (s, (C=C*H*)$_6$), 5.73 (d, J = 15.9 Hz, ½(C*H*$_2$Ph)$_6$), 5.41-5.33 (m, *H*-C(2)$_6$, *H*-C(3)$_6$ and *H*-C(4)$_6$), 5.25-5.20 (m, ½(C*H*2Ph)$_6$), 4.75 (d, *J* = 9.6 Hz, *H*-C(1)$_6$), 4.28 (dd, *J* = 5.0, 12.5 Hz, *H*-C(6)$_6$), 4.14 (dd, *J* = 1.9, 12.5 Hz, *H*-C(6)$_6$), 3.88 (ddd, *J* = 2.3, 5.0, 10.0 Hz, *H*-C(5)$_6$), 2.06 (s, (OAc)$_6$), 2.05 (s, (OAc)$_6$), 2.04 (s, (OAc)$_6$), 1.88 (s, (OAc)$_6$). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.6, 170.0, 169.5, 144.7, 137.1, 124.0, 76.3, 73.6, 73.1, 71.5, 68.5, 62.2, 47.6, 20.6. IR (film) ν = 2954, 2358, 1744, 1364, 1221, 1035, 914 cm^{-1}.

TGB (7)



A solution of **10** (0.160 g, 0.118 mmol) in 2.8 mM K_2CO_3 MeOH solution (10 mL) was stirred at reflux temperature for 1 h. Water and amberlite IR120 were added and the solution was stirred for another ½ h. The beads were filtered off and the product was concentrated *in vacuo*. This yielded **7** (0.101 g, 0.119 mmol, quant.) as a white solid. R_f (MeOH/CH_2Cl_2 1:9) = 0.0. ^{1}H-NMR (400 MHz, D_2O) δ = 7.92 (s, 3 H), 5.79 (s, 6 H), 4.53 (d, *J* = 9.7 Hz, 3 H), 3.89 (dd, *J* = 1.7, 12.4 Hz, 3 H), 3.75 (dd, *J* = 5.1, 12.4 Hz, 3 H), 3.69 (d, *J* = 9.5 Hz, 3 H), 3.63 (d, *J* = 8.7 Hz, 3 H), 3.58 (dd, *J* = 2.3, 5.5 Hz, 3 H), 3.55 (d, *J* = 8.8 Hz, 3 H), 2.32 (s, 9H). ^{13}C-NMR (75 MHz, D_2O) δ = 144.3, 140.0, 129.4, 123.9, 79.4, 76.6, 73.0, 72.3, 69.0, 60.3, 48.8, 15.2. HRMS (ESI) *m/z* calculated for $C_{36}H_{51}N_9Na_1O_{15}$ $(M+Na)^+$ 872.34023, found 872.33954.

HGB (8)



A solution of **11** (0.15 g, 0.058 mmol) in 2.8 mM K_2CO_3 MeOH solution (10 mL) was stirred at reflux temperature for 1½ h. Water and amberlite IR120 were added and the solution was stirred for another ½ h. The beads were filtered off and the product was concentrated *in vacuo*. This yielded **8** (90 mg, 0.059 mmol, quant.) as a white solid. R_f (MeOH/CH_2Cl_2 1:9) = 0.0. ^{1}H-NMR (400 MHz, D_2O) δ = 7.87 (s, Tr*H*), 6.00 (s, CH_2Ph), 4.46 (d, *J* = 9.3 Hz, *H*-C(1)), 3.93 (dd, *J* = 2.0, 12.5 Hz, *H*-C(6)), 3.77 (dd, *J* = 5.3, 12.4 Hz, *H*-C(6)), 3.61-3.51 (m, 24H). ^{13}C-NMR (75 MHz, D_2O) δ = 144.8, 136.9, 123.9, 79.5, 76.5, 73.0, 72.5, 69.1, 60.5, 48.1. IR (film) ν = 3321, 2876, 2103, 1048 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{60}H_{84}N_{18}Na_1O_{30}$ $(M+Na)^+$ 1559.54984, found 1559.54258.

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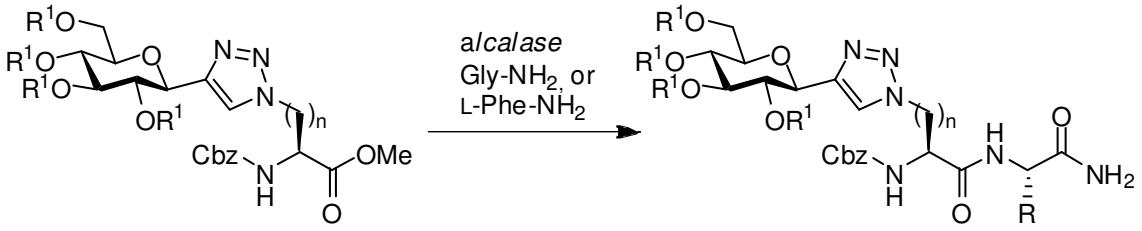
6 Chemoenzymatic peptide coupling in the synthesis of triazole-linked glycopeptides

Abstract

Chemoenzymatic peptide coupling of azide- or acetylene-containing amino acid methyl esters with natural amino acid amides under the influence of *alcalase* was investigated. Condensation was found to proceed cleanly and in high yield. Incorporation of glycoamino acid esters in peptides is investigated for a range of different protective groups and side-chains, and was most successful for glycoamino acid esters with acetyl protecting groups (R^1 = Ac) and a short side-chain (n = 1).



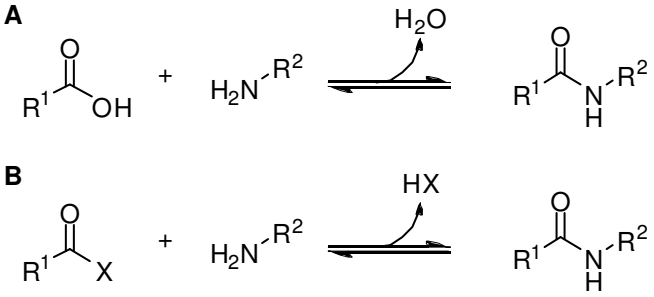
Part of this chapter has been published: a) Groothuys, S.; Kuijpers, B. H. M.; Quaedflieg, P. J. L. M.; Roelen, H. C. P. F.; Wiertz, R. W.; Blaauw, R. H.; van Delft, F. L.; Rutjes, F. P. J. T., *Synthesis* **2006**, 3146. b) Kuijpers, B. H. M.; Groothuys, S.; Hawner, C.; ten Dam, J.; Quaedflieg, P. J. L. M.; Schoemaker, H. E.; van Delft, F. L.; Rutjes, F. P. J. T. *Org. Process Res. Dev.* **2008**, 12, 503

6.1 Introduction

The current state-of-the-art of peptide synthesis typically involves a reiterative deprotection-coupling protocol starting from the amino acid at the C-terminus of a desired peptide. Such an approach has proven to be highly valuable and peptides numbering over one hundred residues can thus be prepared on the solid phase. Conceptually quite distinct, chemoenzymatic peptide synthesis has received relatively sparesome but increasing interest over the last decade,[1, 2] and is characterized by a number of specific advantages over chemical peptide synthesis: (a) chemoenzymatic peptide coupling reactions are always free from racemization, (b) side-chain protection can mostly be omitted, (c) the coupling reagent (enzyme) can be recycled efficiently, (d) the process is environmentally friendly, (e) the reaction proceeds under mild conditions and (f) can be readily scaled up. On the down-side, there lies a great challenge in chemoenzymatic peptide synthesis involving incorporation of proline[3] and/or non-proteinogenic amino acids such as D-, β- and unnatural amino acids.[4] In Chapter 3 we showed that triazole-linked glycoamino acids and peptides can be acquired by a combination of Cu(I)-catalyzed azide acetylene 1,3-dipolar condensation (CuAAC) and a typical diimide-mediated peptide coupling.[5] We have now broadened the repertoire of preparation of triazole-linked glycopeptides by elongation of glycoamino acids with proteinogenic amino acids via both conventional solution-phase chemical peptide synthesis and chemoenzymatic procedures. Under the influence of *alcalase*, not only acetyleno- and azidoalkylamino acid methyl esters were found to act as acyl donors for condensation with *N*-unprotected amino acid amides, but also triazole-linked glycoamino acids could be successfully coupled.

In a typical chemoenzymatic peptide synthesis protocol,[2] an *N*-protected amino acid (bearing a free carboxy group) is condensed with a *C*-protected amino acid. Unfortunately, since the reaction is equilibrium-controlled (Scheme 6.1A), the *thermodynamically* more stable starting materials (in other words, the hydrolysis products) are usually favored unless the desired condensation product precipitates or is otherwise removed from the solution. An alternative *kinetically* controlled approach (Scheme 6.1B) involves *C*-activation of an *N*-protected amino acid.[6] Such an approach is potentially more favorable since the yield is now predominantly

determined by enzymatic properties such as substrate specificity and the ratio of synthesis *vs.* hydrolysis.[2] The most suitable enzymes for chemoenzymatic peptide synthesis include the majority of the commercially available serine, metallo-, and aspartate endo- and exoproteases.[7,8,9]

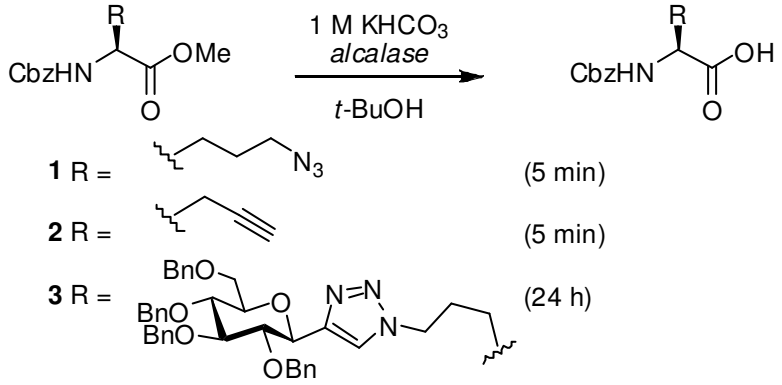


Scheme 6.1 Equilibrium-controlled (**A**) and kinetically controlled (**B**) enzymatic peptide synthesis (R^1-COOH = carboxy component; R^1-CO-X = slightly activated carboxy component; H_2N-R^2 = amino component; HX = leaving group)

6.2 Peptide coupling

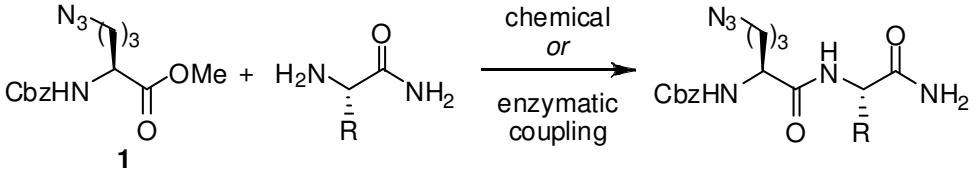
In order to effect enzymatic coupling of a neutral and bulky *N*-protected unnatural amino acid such as azidopropylglycine methyl ester (**1**) and propargylglycine methyl ester (**2**), with a proteinogenic *C*-protected amino acid, the enzymes that were considered involved the typical *subtilisin* or *carboxypeptidase Y*.[1] However, inspired by the work of Chen *et al.*,[7] it was instead decided to focus attention on cheap and industrially available *alcalase*, a proteolytic enzyme mixture produced by *Bacillus licheniformis* (containing *subtilisin Carlsberg* as the major enzyme component), with high activity and stability in alcoholic solvents. Thus, in order to determine whether **1** and **2** are suitable substrates for *alcalase*, initial experiments involved hydrolysis to the free acids in a 1:1 mixture of *t*-butyl alcohol and 1 M aqueous potassium bicarbonate (Scheme 6.2).

It was observed that both **1** and **2** were completely hydrolyzed after 5 min, but hydrolysis of the triazole-linked glycoamino acid methyl ester **3** was much slower (complete conversion after 24 h with a 5-fold excess of *alcalase*). Most likely, the bulky glycosidic side-chain of **3** is not well accommodated in the active site of the enzyme. Nevertheless, the successful chemoenzymatic hydrolysis under the action of *alcalase* was a clear indication the amino acid esters were suitable substrates for the enzyme.



Scheme 6.2 *Alcalase*-induced hydrolysis of amino acid methyl esters.

Table 6.1 Chemical and chemoenzymatic condensation of azidopropylglycine methyl ester **1**

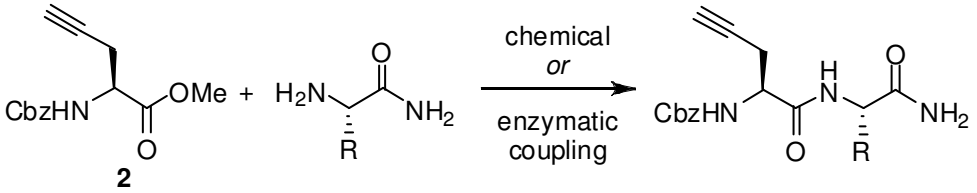


entry	R	conditions	dipeptide	yield
1	H	chemical[a]	**4**	80%
2	H	chemoenzymatic[b]		95%
3	CH_2Ph	chemical[a]	**5**	69%
4	CH_2Ph	chemoenzymatic[b]		77%

[a] 1. NaOH, THF; 2. EDCI, HOAt, DIPEA, DMF
[b] a*lcalase*, *t*-BuOH, DMF, 35 °C

In the next step, *alcalase*-promoted condensation of the ornithine-derived donor **1** was explored with amino acid nucleophiles glycine amide and phenylalanine amide, under anhydrous conditions (Table 6.1). We were delighted to find that the respective dipeptides **4** and **5** were formed in high yields (entries 2 and 4) by chemoenzymatic condensation, comparing favorably to chemical coupling with EDCI (entries 1 and 3). Furthermore, the resulting dipeptides were formed with exclusive diastereoselectivity, *i.e.* without detectable racemization.

Table 6.2 Chemical and chemoenzymatic condensation of propargylglycine methyl ester **2**

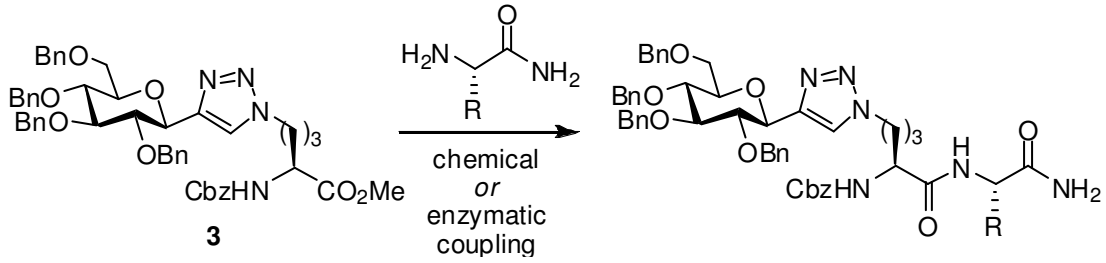


entry	R	conditions	dipeptide	yield
1	H	chemical[a]	**6**	60%
2	H	chemoenzymatic[b]		80%
3	CH_2Ph	chemical[a]	**7**	55%
4	CH_2Ph	chemoenzymatic[b]		79%

[a] 1. NaOH, THF; 2. EDCI, HOAt, DIPEA, DMF
[b] *alcalase*, *t*-BuOH, DMF, 35 °C

Similarly, dipeptides **6** and **7** were formed successfully by *alcalase*-mediated coupling of propargylglycine **2** to glycine amide and phenylalanine amide, respectively (Table 6.2, entries 2 and 4). Again, the isolated yields compare favorably to the same condensations executed under the action of EDCI in DMF.

Table 6.3 Chemical and chemoenzymatic condensation of T1P($GlcBn_4$) methyl ester **3**



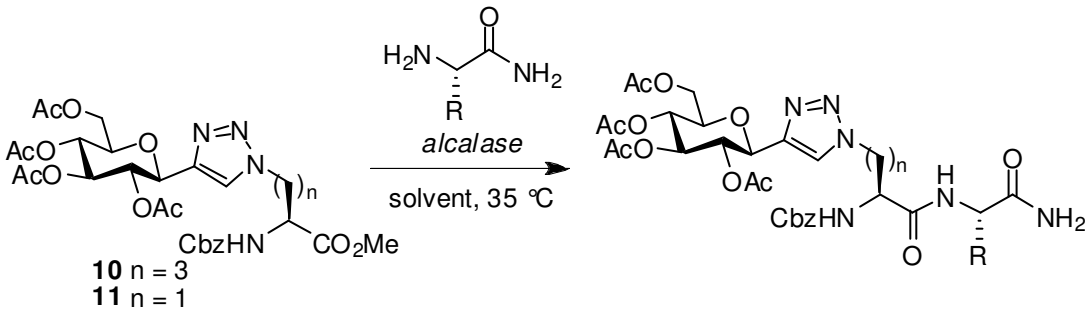
entry	R	conditions	dipeptide	yield
1	H	chemical[a]	**8**	82%
2	H	chemoenzymatic[b]		10%
3	CH_2Ph	chemical[a]	**9**	86%
4	CH_2Ph	chemoenzymatic[b]		0% (40%)[c]

[a] 1. NaOH, THF; 2. EDCI, HOAt, DIPEA, DMF
[b] *alcalase*, *t*-BuOH, DMF, 35 °C
[c] conversion of acyl donor as determined by HPLC

Not unexpectedly, under similar conditions the condensation of glycine amide and phenylalanine amide with the sterically hindered tetrabenzylated glycoamino acid

methyl ester **3** proceeded extremely slow (40% conversion of **3** after 8 days) and did not lead to a satisfactory yield of the desired dipeptides **8** and **9**[10] (Table 6.3, entries 2 and 4). In contrast, chemical amide bond formation was not sensitive to the sterical bulk of the glycoamino acid and proceeded without incidence in good yield (entries 1 and 3).

Table 6.4 Chemoenzymatic condensation of T1P and T1M methyl esters **10** and **11**



entry	R	n	solvent	time	ester (%)[a]	impurities (%)[a]	peptide (%)[a]	
1	H	3	*t*-amyl-OH	24 h	87	4	**12**	9
2				48 h	84	4		12
3				9 d	57	9		34
4	CH_2Ph	3	*t*-amyl-OH	24 h	67	10	**13**	23
5				48 h	54	19		27
6				9 d	4	88		8
7	H	1	*t*-BuOH, DMF	20 h	100	0	**14**	0
8				4 d	58	19		23
9				8 d	42	21		37
10				14 d	16	26		58
11				15 d	0	34		66
12	CH_2Ph	1	*t*-BuOH, DMF	2 h	93	-	**15**	7
13				20 h	61	-		39
14				4 d	6	-		94
15				6 d	6	-		94

[a] Based on HPLC analysis

Replacement of the bulky benzyl protecting groups to less hindered acetyl functions was expected to give some increase in enzyme activity. Indeed, as shown in Table 6.4 (entries 1-3 and 4-6), a small increase in yield with respect to benzyl protection is accomplished, although the isolated yields were still not satisfactory.

Much to our surprise, altering the glycoamino acid from triazolylpropylglycine (T1P; n = 3) to a ^{C}TGA with a shorter side-chain (T1M; n = 1) and coupling with glycine amide and phenylalanine amide now gave the desired dipeptides with significantly enhanced yields after prolonged stirring (**14**, entries 7-11 and **15**, entries 12-15, respectively). Interestingly, the coupling of phenylalanine amide with tetraacetylated glycoamino acid **11** proceeded in near quantitative yield to form **15**, while the coupling with glycine amide to form **14** was a little more troublesome but still led to the desired dipeptides in satisfactory yield.

The latter chemoenzymatic coupling was followed closely by RP-HPLC as visualized in Figure 6.1. From the graph, it becomes clear that although complete conversion of T1M **11** is attained with 66% formation of the dipeptide **14**, an increasing amount of an impurity is also observed, reaching 34%after 15 days of stirring.

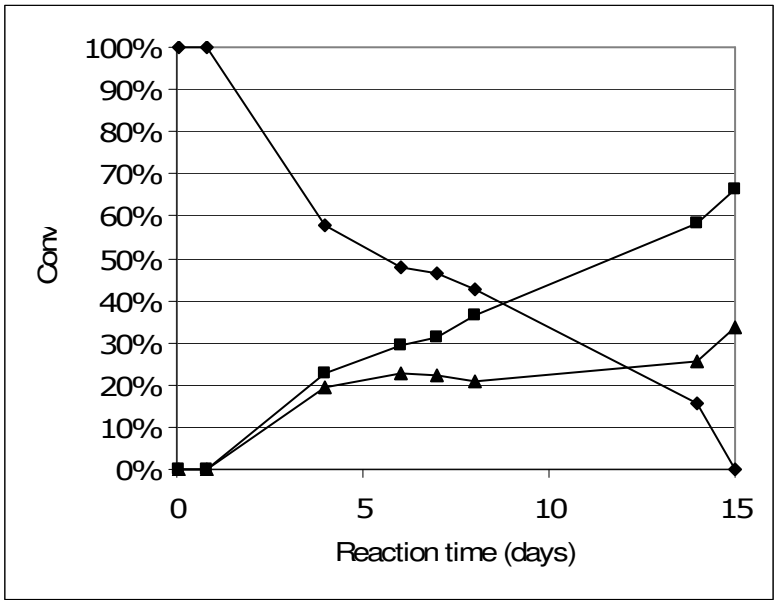


Figure 6.1 Chemoenzymatic conversion of **11** (♦) to **14** (■) and the formation of unidentified impurities (▲)

Unfortunately, the identity of the impurity could not be established, although it was clear that chemoenzymatic hydrolysis of starting material was not an issue. Since glycine amide was added in excess to the reaction mixture, formation of a tripeptide (T1M(GlcAc$_4$)-Gly-Gly-NH$_2$) might be one of the possibilities. The dipeptides **14** and **15** were isolated, after crystallization, in 65% and 98% yield respectively.

6.3 Conclusions

A chemoenzymatic coupling strategy was successfully applied to the preparation of triazole-linked glycodipeptides. In particular, *alcalase*-induced condensation of an acetyleno- or azidoalkylamino acid methyl ester with an amino acid amide proceeded with high conversion and good yield. For the chemoenzymatic condensation of carbohydrate-substituted triazolylamino acids (^{C}TGAs) optimal results were obtained with a carbohydrate component protected with acetyl functions rather than benzyl groups, an observation that may be rationalized in terms of either lipophilicity or steric factors, or a combination thereof. Finally, it was found that amino acid methyl esters with a shorter side-chain length afforded the desired dipeptides with good to excellent yield.

6.4 Acknowledgements

Dr. Harlof Roelen, Christine Hawner and Roel Wiertz (DSM, Geleen, The Netherlands) are kindly acknowledged for help with the chemoenzymatic peptide coupling. Jaen Adrien (DSM, Geleen, The Netherlands) is acknowledged for analytical support. Dr. Peter Quaedflieg (DSM, Geleen, The Netherlands) is acknowledged for his hospitality and fruitful discussions.

6.5 Experimental section

General information

Before use in the coupling reactions, L-Phe-NH$_2$ was dried by coevaporation with DMF (2 × 5 mL per mmol) and Gly-NH$_2$·HCl was first neutralized by treatment with 1 equiv of NaOMe (25% in MeOH) in EtOH (0.5 M), subsequently isolated as solid (Gly-NH$_2$) by filtration and evaporation, and repeated coevaporation with toluene. *t*-BuOH was dried by distillation under reduced pressure and kept under nitrogen. *Alcalase* was obtained from Novo Industrial (Denmark) as a brown aqueous liquid. For the hydrolysis experiments this enzyme solution was used as such. For the

(anhydrous) peptide coupling reactions the water in the *alcalase* solution was removed using the method of Chen *et al.*[7] As a matter of fact, the aqueous *alcalase* (0.5 mL) and absolute ethanol (1.0 mL) were mixed in an Eppendorf cup and the resulting suspension was agitated on a Vortex mixer for 5 min and centrifuged (3000 rpm) for 10 min to spin down the enzyme. The supernatant was decanted and the enzyme resuspended in absolute EtOH (1.0 mL), agitated for 5 min on a Vortex mixer and spun down by centrifugation. This procedure was repeated once with absolute EtOH (1.0 mL) and finally once with dry *t*-BuOH (1.0 mL). The resulting enzyme was resuspended in *t*-BuOH (1.0 mL) for use in the chemoenzymatic peptide coupling reactions. All other commercially available reagents were used as received. HPLC analyses were performed with an Inertsil RP-18 column (25 × 0.46 cm) (RP-18, 5 μm). For further general information, see section 2.6.

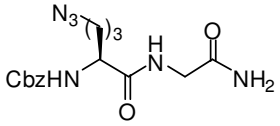
General procedure for chemoenzymatic peptide coupling

The *N*-Cbz-protected amino acid methyl ester (1 mmol) was dried by coevaporation with toluene and dissolved in *t*-BuOH (0.25 M). After adding a solution of Phe-NH_2 or Gly-NH_2 (1.5 mmol) in DMF (1 M), the reaction mixture was stirred at 35 °C. Subsequently, the *alcalase* suspension in *t*-BuOH (1 mL) was added and the reaction mixture was stirred at 35 °C. Samples were taken at regular time intervals and analyzed by HPLC. After almost complete conversion of the methyl ester, the reaction mixture was concentrated. The residue was redissolved in EtOAc (50 mL) and H_2O (20 mL), to which a few drops of 1 N aqueous HCl were added. The aqueous layer was extracted with EtOAc (30 mL) and the combined organic phase was washed with aqueous $KHCO_3$ (1 M, 40 mL), aqueous HCl (1 N, 40 mL) and brine (40 mL), dried (Na_2SO_4) and concentrated. Analytically pure samples were obtained by recrystallization.

General procedure for chemical peptide coupling

Cbz-amino acid (1 mmol) was dissolved in DMF (2.5 mL). To the clear cooled (0-5 °C) solution was added DIPEA (0.35 mL, 2.1 mmol) followed by EDCI (211 mg, 1.1 mmol) and HOAt (150 mg, 1.1 mmol) and the mixture was stirred for at least 0.5 h at 0-5°C. Subsequently, a solution of Phe-NH_2 or Gly-NH_2 (1.1 mmol) in DMF (2.5 mL) was added and the mixture was stirred for 1 h at 0-5 °C. The mixture was warmed to rt and stirred overnight. TLC analysis (EtOAc) showed complete conversion of the carboxylic acid starting material. The reaction mixture was partitioned between EtOAc (75 mL) and H_2O (15 mL) to which a few drops of 1 N aqueous HCl were added. The aqueous layer was extracted with EtOAc (40 mL) and the combined organic phase was washed with 0.5 N aqueous HCl (40 mL), 1 M aqueous $KHCO_3$ (40 mL) and brine (40 mL), dried (Na_2SO_4) and concentrated.

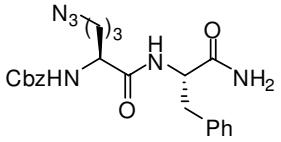
Cbz-L-Orn(N_3)-Gly-NH_2 (4)



Chemical coupling, yield: 277 mg (80%). Chemoenzymatic coupling, yield: 325 mg (93%). R_f (EtOAc) = 0.39. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.36-7.30 (m, 5 H), 5.13 (d,

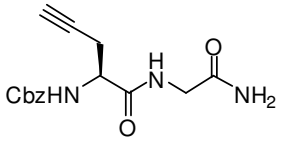
J = 12.3 Hz, 1 H), 5.08 (d, *J* = 12.3 Hz, 1 H), 4.13-4.10 (m, 1 H), 3.93 (d, *J* = 17.1 Hz, 1 H), 3.81 (d, *J* = 17.1 Hz, 1 H), 3.34-3.31 (m, 2 H), 1.94-1.62 (m, 4 H). IR (film) ν = 3343, 2483, 2241, 2072, 1662 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{15}H_{20}N_6O_4Na$ $(M+Na)^+$ 371.14437, found 371.14351.

Cbz-L-Orn(N_3)-L-Phe-NH_2 (5)



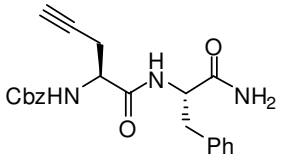
Chemical coupling, yield: 302 mg (69%). Chemoenzymatic coupling, yield: 339 mg (77%). R_f (2/1 EtOAc/heptane) = 0.29. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.38-7.17 (m, 10 H), 5.10 (d, *J* = 12.3 Hz, 1 H), 5.06 (d, *J* = 12.3 Hz, 1 H), 4.63 (dd, *J* = 5.9, 8.4 Hz, 1 H), 4.04 (dd, *J* = 5.7, 8.0 Hz, 1 H), 3.25-3.16 (m, 3 H), 2.93 (dd, *J* = 5.5, 8.6 Hz, 1 H), 1.74-1.44 (m, 4 H). IR (film) ν = 3304, 2470, 2098, 1679, 1640 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{22}H_{26}N_6O_4Na$ $(M+Na)^+$ 461.19132, found 461.19216.

Cbz-L-propagylglycine-Gly-NH_2 (6)



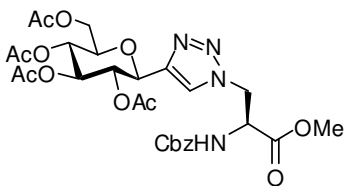
Chemical coupling, yield: 181 mg (60%). Chemoenzymatic coupling, yield: 244 mg (80%). R_f (EtOAc) = 0.39. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.34-7.27 (m, 5 H), 5.12 (d, *J* = 12.3 Hz, 1 H), 5.08 (d, *J* = 12.3 Hz, 1 H), 4.28 (t, *J* = 6.5 Hz, 1 H), 3.91 (d, *J* = 17.1 Hz, 1 H), 3.81 (d, *J* = 17.1 Hz, 1 H), 2.71 (ddd, *J* = 2.6, 6.2, 17.0 Hz, 1 H), 2.65 (ddd, *J* = 2.6, 6.8, 17.0 Hz, 1 H), 2.19 (t, *J* = 2.6 Hz, 1 H). IR (film) ν = 3291, 3066, 3036, 2941, 2409, 1658, 1532 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{15}H_{17}N_3O_4Na$ $(M+Na)^+$ 326.11168, found 326.11042.

Cbz-L-propargylglycine-L-Phe-NH_2 (7)



Chemical coupling, yield: 536 mg (55%). Chemoenzymatic coupling, yield: 312 mg (79%). R_f (2/1 EtOAc/heptane) = 0.25. ^{1}H-NMR (400 MHz, $CDCl_3$/MeOD) δ = 7.39-7.20 (m, 10 H), 5.10 (s, 2 H), 4.65-4.62 (m, 1 H), 4.28-4.25 (m, 1 H), 3.20-3.15 (m, 1 H), 3.00-2.95 (m, 1 H), 2.65-2.54 (m, 2 H), 2.12 (br s, 1 H). IR (film) ν = 3351, 2474, 2220, 2064, 1671, 1632 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{22}H_{23}N_3O_4Na$ $(M+Na)^+$ 416.15863, found 416.15939.

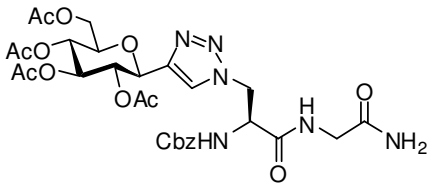
Cbz-L-T1M{4-[β-D-Glc($Ac)_4$]}-OMe (11)



To a solution of Ac_4Glc-acetylene (1.0 g, 2.8 mmol) and Cbz-L-Dap(N_3)-OMe (0.78 g, 2.8 mmol) in *t*-BuOH (15 mL) and MeCN (2 mL) was added a mixture of $Cu(OAc)_2$ (112 mg, 0.56 mmol) and sodium ascorbate (218 mg, 1.1 mmol) in water (15 mL), and the reaction was stirred overnight at rt, after the addition of EtOAc (250 mL) the mixture was washed with H_2O (2 × 50 mL), brine (2 × 50 mL), dried (Na_2SO_4) and concentrated. Purification by gradient flash chromatography (MeOH in CH_2Cl_2 0 → 10%) yielded product **11** (1.7 g, mmol, 96%) as a white solid. R_f (EtOAc) = 0.74. ^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD) δ = 7.75 (s, 1 H); 7.41-7.29 (m, 5 H); 5.37 (t, *J* = 9.4 Hz, 1 H); 5.25 (t,

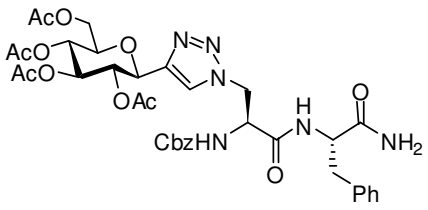
J = 9.7 Hz, 1 H); 5.18 (dd, *J* = 10.0, 9.4 Hz, 1 H); 5.12 (s, 2 H); 4.91-4.71 (m, 4 H); 4.28 (dd, *J* = 12.5, 4.9 Hz, 1 H); 4.13 (dd, *J* = 12.5, 2.2 Hz, 1 H); 3.94 (ddd, *J* = 10.1, 4.8, 2.2 Hz, 1 H); 3.79 (s, 3 H); 2.07 (s, 3 H); 2.05 (s, 3 H); 2.02 (s, 3 H); 1.86 (s, 1 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.5, 170.0, 169.7, 169.4, 169.0, 155.6, 144.3, 135.8, 128.5, 128.2, 128.0, 123.2, 76.2, 73.7, 73.1, 71.3, 68.3, 67.3, 62.0, 53.9, 53.1, 50.7, 20.6, 20.5, 20.3. IR (film) ν = 3356, 3140, 2950, 2885, 2250, 1748, 1532 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{28}H_{34}N_4NaO_{13}$ $(M+Na)^+$ 657.20201, found 657.19955.

Cbz-L-T1M{4-[β-D-Glc(Ac)$_4$]}-Gly-NH$_2$ (14)



Applying general method (with 0.37 mmol **11**, and 5 equiv. Gly-NH$_2$) for chemoenzymatic peptide coupling gave **14** (165 mg; 65%). R_f (EtOAc) = 0.25. ^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD) δ = 7.93 (s, 1 H), 7.33 (m, 5 H), 5.38 (t, *J* = 9.4 Hz, 1 H), 5.28 (t, *J* = 9.7 Hz, 1 H), 5.17 (t, *J* = 9.7 Hz, 1 H), 5.10-5.04 (m, 2 H), 4.90 (dd, *J* = 13.8, 4.5 Hz, 1 H), 4.81-4.64 (m, 3 H), 4.28 (dd, *J* = 12.4, 4.8 Hz, 1 H), 4.12 (dd, *J* = 12.4, 2.1 Hz, 1 H), 3.98 (ddd, *J* = 10.0, 4.8, 2.2 Hz, 1 H), 3.92 (d, *J* = 17.1 Hz, 1 H), 3.83 (d, *J* = 17.1 Hz, 1 H), 2.05 (s, 3 H), 2.03 (s, 3 H), 2.00 (s, 3 H), 1.85 (s, 3 H). ^{13}C-NMR (75 MHz, CD_3OD) δ = 174.0, 172.3, 171.7, 171.3, 171.0, 145.4, 137.7, 129.5, 129.1, 129.0, 125.8, 77.1, 75.4, 73.6, 72.9, 69.8, 68.1, 63.4, 56.2, 51.7, 43.2, 20.6, 20.5. IR (film) ν = 3330, 2955, 1744, 1671, 1524 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{29}H_{36}N_6NaO_{13}$ $(M+Na)^+$ 699.22380, found 699.22173.

Cbz-L-T1M{4-[β-D-Glc(Ac)$_4$]}-L-Phe-NH$_2$ (15)



Applying general method (with 0.39 mmol **11**, and 5 equiv. Phe-NH$_2$) for chemoenzymatic peptide coupling gave **15** (0.30 g; 98%).R_f (EtOAc) = 0.25. ^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD) δ = 7.81 (s, 1 H), 7.41-7.14 (m, 10 H), 5.37 (t, *J* = 9.4 Hz, 1 H), 5.24 (t, *J* = 9.7 Hz, 1 H), 5.17 (t, *J* = 9.7 Hz, 1 H), 5.06 (s, 2 H), 4.81-4.74 (m, 2 H), 4.66-4.60 (m, 3 H), 4.28 (dd, *J* = 12.5, 4.9 Hz, 1 H), 4.12 (dd, *J* = 12.4, 2.1 Hz, 1 H), 3.96 (ddd, *J* = 10.1, 4.7, 2.1 Hz, 1 H), 3.16 (dd, *J* = 13.9, 5.9 Hz, 1 H), 2.95 (dd, *J* = 13.9, 8.3 Hz, 1 H), 2.06 (s, 3 H), 2.03 (s, 3 H), 2.01 (s, 3 H), 1.85 (s, 3 H). ^{13}C-NMR (75 MHz, CD_3OD) δ = 175.1, 172.1, 171.5, 171.0, 170.8, 170.0, 157.5, 145.0, 137.7, 137.3, 130.0, 129.3, 129.3, 129.0, 128.8, 128.6, 127.6, 125.4, 76.9, 75.1, 73.5, 72.6, 69.5, 67.9, 63.2, 55.7, 55.4, 51.4, 38.5, 20.7, 20.6. IR (neat) ν = 3417, 3283, 2950, 1748, 1697, 1636, 1532 cm^{-1}. HRMS (ESI) *m/z* calculated for $C_{36}H_{42}N_6NaO_{13}$ $(M+Na)^+$ 789.27075, found 789.26506.

6.6 References

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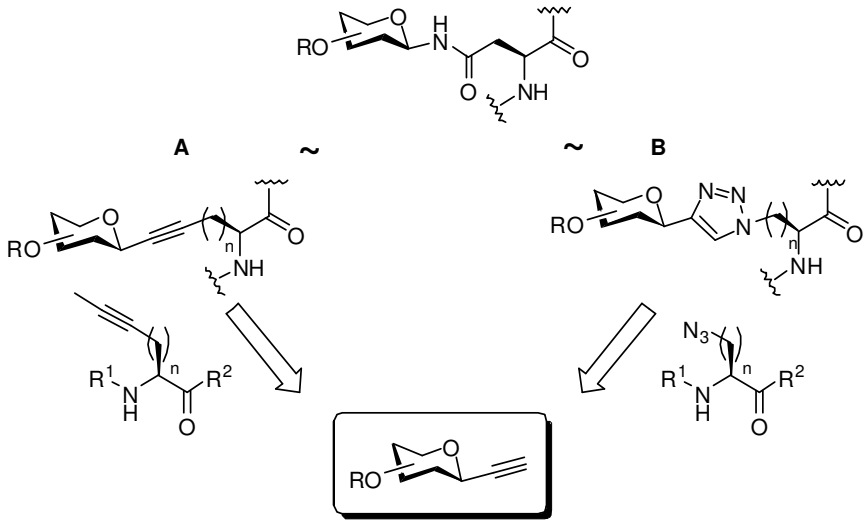
10. For the experimental section see chapter 3, compunds **39** and **40**, respectively

Summary

Glycopeptides – conjugates of peptides with *O*- or *N*-glycans – are biologically active compounds which play an essential role in nature. However, the exact biological role and mode of action of these glycopeptides is still largely unsolved. This calls for the synthesis of specific glycopeptide fragments and application in biological systems in order to gain further inside into these matters. Since the natural sugar-peptide acetal linkage is readily cleaved by hydrolytic enzymes, it is also attractive to prepare glycopeptide mimics containing stable acetal isosteres. An example of such an isosteric replacement is the substitution of the native linkage by the unnatural *C*-linker as shown in Scheme 1.



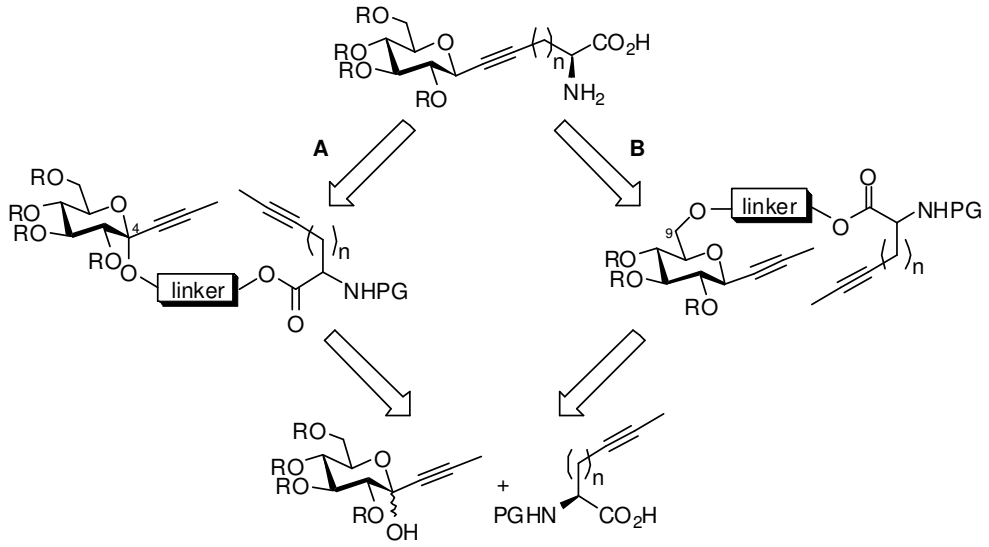
Scheme 1 Isosteric replacement of the native linkage in glycoamino acids and peptides by alkyne (**A**) and triazole (**B**) linkers, which both can be made from glycoacetylenes

Glycoacetylenes, carbohydrate derivatives functionalized with an acetylene group at one of the ring-carbons, constitute a compound class that can be used as a versatile starting point for synthesizing *C*-glycosides, such as *C*-glycoamino acids and peptides. The research described in this thesis aims at using glycoacetylenes for the synthesis of different classes of stable glycopeptide mimics and their evaluation in a biological setting.

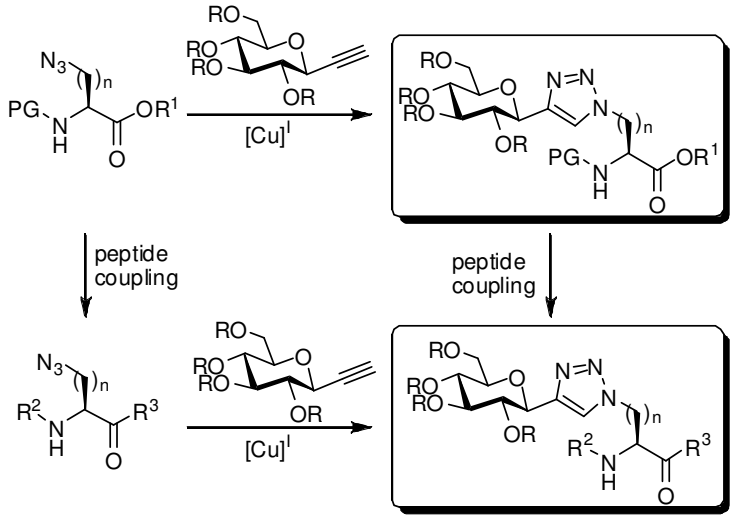
Chapter 1 provides an overview of the synthesis of glycoacetylenes by a range of different methodologies. In general, the methods leading to glycoacetylenes comprise the application of nucleophilic metal acetylide addition to carbohydrate electrophiles, in particular at the anomeric carbon. A sophisticated choice of metal counterions (*e.g.* magnesium, lithium, zinc and tin) and conditions leads to the selective formation of either α- or β-configured glycoacetylenes. Glycoacetylenes can be applied in different ways for the synthesis of *C*-glycoamino acids. Examples given involve the synthesis

of alkane-, alkyne-, tryptophan-, and triazole-linked glycoamino acids, as well as conformationally restricted glycoamino acids.

In Chapter 2, the synthesis of alkyne-linked glycoamino acids via ring-closing alkyne metathesis is investigated. Two different strategies are described to attach alkynylamino acids to an alkynylsugar, which may proceed either via a linker at O-4 (Scheme 2, path A) or at O-9 (path B) of the alkynylsugar. Subsequent ring-closing alkyne metathesis of the O-4–linked diynes failed to proceed, but alkyne-linked glycoamino acids of varying chain length were effectively synthesized via attachment at O-9.



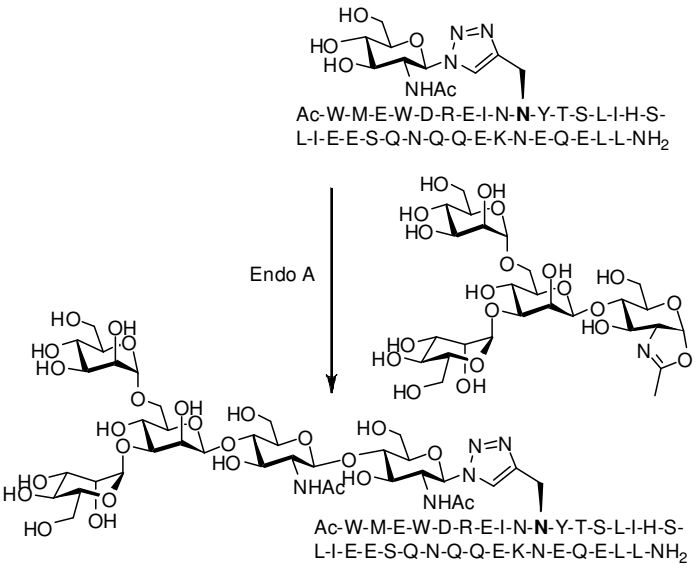
Scheme 2 Retrosynthesis of alkyne-linked glycoamino acid, by means of RCAM via O-4 linkage (Path **A**) or O-9 linkage (Path **B**)



Scheme 3 Synthesis of triazole-linked glycoamino acids and peptides

Chapter 3 delineates methodology for the synthesis of triazole-linked glycoamino acids and peptides based on the copper(I)-catalyzed azide acetylene cycloaddition of glycoacetylenes with azidoamino acids (Scheme 3). The resulting triazolyl glycoamino acids appeared suitable building blocks for the assembly of isosteric glycopeptides. An alternative route to such triazolyl glycopeptides was also developed by late-stage introduction of the carbohydrate portion, involving similar cycloaddition of glycoacetylenes with azidopeptides.

Chapter 4 details the chemoenzymatic transglycosylation of *C*- and *N*-triazole-linked glycopeptides with a carbohydrate oxazoline donor under the action of the enzyme Endo A (Scheme 4). While the conversion of a small *C*-triazolyl glycodipeptide stopped at a conversion of 50%, the corresponding *N*-triazolyl derivative was converted to the corresponding pentasaccharide with an efficiency of 80%. Next, the same chemoenzymatic glycosylation procedure was successfully applied to a more complex glycopeptide, namely triazole-linked *N*-GlcNAc-modified peptide C34, which is present in the C-terminal fragment of HIV-1 gp41. The resulting novel triazole-linked glycopeptide appeared fully resistant to peptide *N*-glycanase (PNGase F), a serum peptidase that effectively digests the native amide-linked glycopeptide.



Scheme 4 Endo A-catalyzed glycosylation of GlcNAc-C34*

Chapter 5 describes the synthesis of oligoglycosides by condensation of glycoacetylenes with tri- and hexaazidohexamethylbenzene in the presence of a copper-wired stirring rod. As depicted in Figure 1, the resulting triazole-linked oligosaccharides appear to form supramolecular gels, presumably by a combination of hydrogen bonding between the saccharide moieties and π-π stacking of the arene moieties, as indicated by molecular modeling. The trisaccharide gel was visualized using SEM, TEM and AFM techniques, all of which manifested the presence of intertwined fibers.

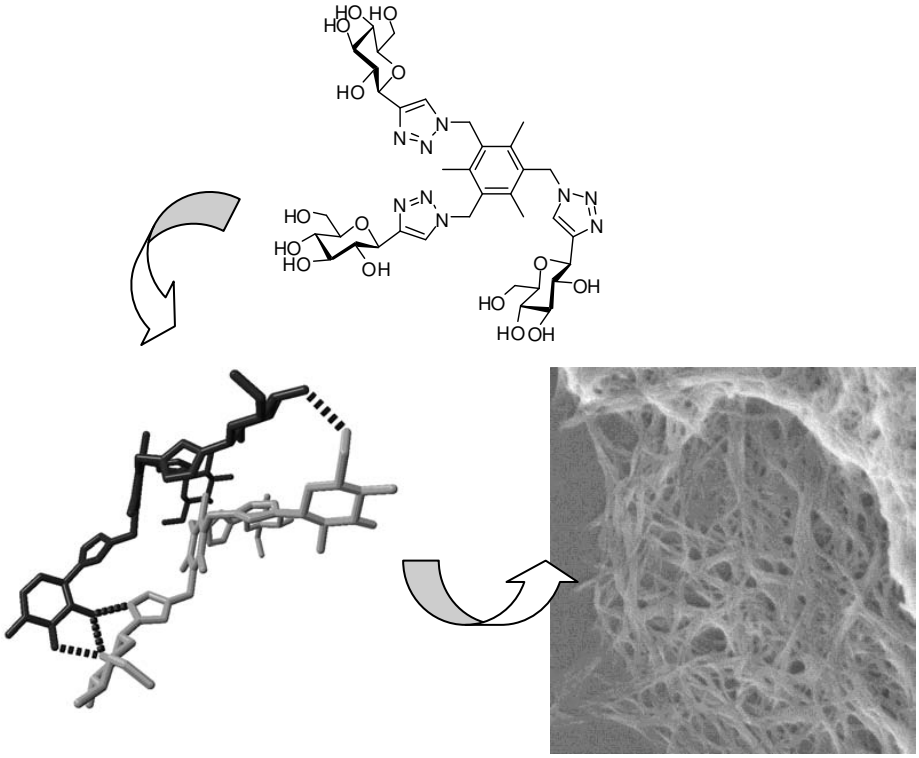
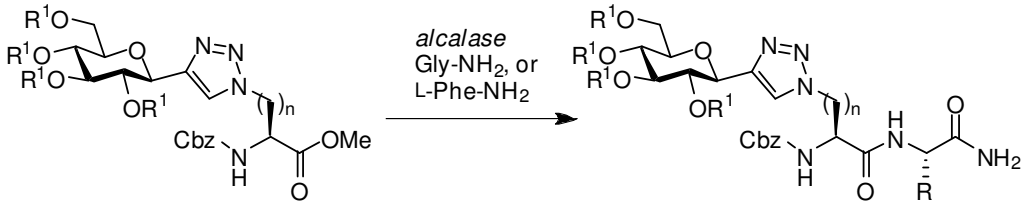


Figure 1 Triazole-linked trisaccharide aggregation to form supramolecular gels

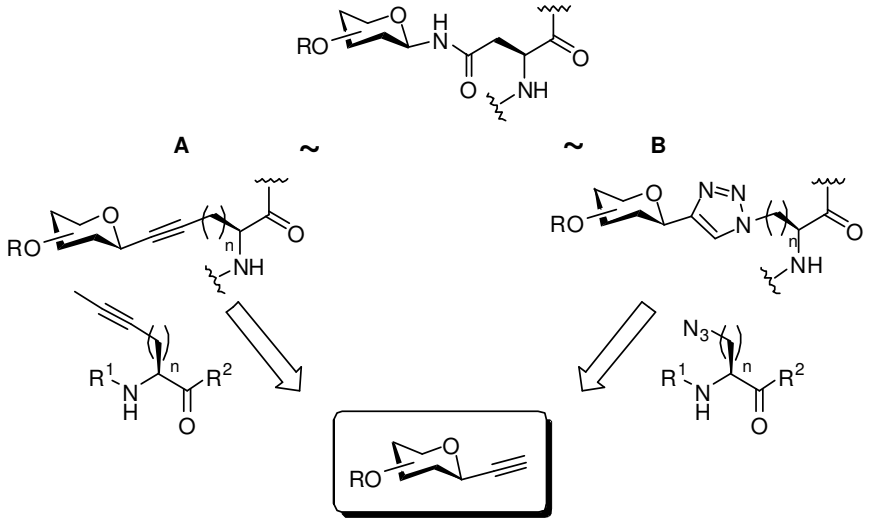
Chemoenzymatic peptide coupling of azide- and acetylene-containing amino acid methyl esters with natural amino acid amides under the influence of the enzyme *alcalase* was investigated in Chapter 6 (Scheme 5). Condensation appeared to proceed cleanly and in high yield. Incorporation of glycoamino acids in peptides was investigated for a range of different protective groups and side-chains, and was most successful for glycoamino acid esters with acetyl protecting groups (R^1 = Ac) in combination with a short side-chain (n = 1).



Scheme 5 Chemoenzymatic coupling of glycoamino acid esters with glycine amide and phenylalanine amide

Samenvatting

Glycopeptiden – conjugaten van peptiden met *O*- of *N*-glycanen – vervullen een belangrijke rol in cellulaire processen. Echter, de exacte biologische werkingsmechanismen van deze glycopeptiden zijn op moleculair niveau nog steeds grotendeels onopgelost. Dit vraagt om de synthese van specifieke glycopeptide fragmenten en hun toepassing in biologische systemen om verdere inzichten te verwerven in hun werking. Aangezien de natuurlijke acetaalbinding tussen suiker en peptide in een cellulaire omgeving snel kan worden verbroken door hydrolytische enzymen, is het aantrekkelijk om glycopeptide mimetica met stabiele acetaal isosteren te synthetiseren. Voorbeelden van een dergelijke isostere vervanging van de natuurlijke linker door stabiele onnatuurlijke linkers zijn weergegeven in Schema 1.



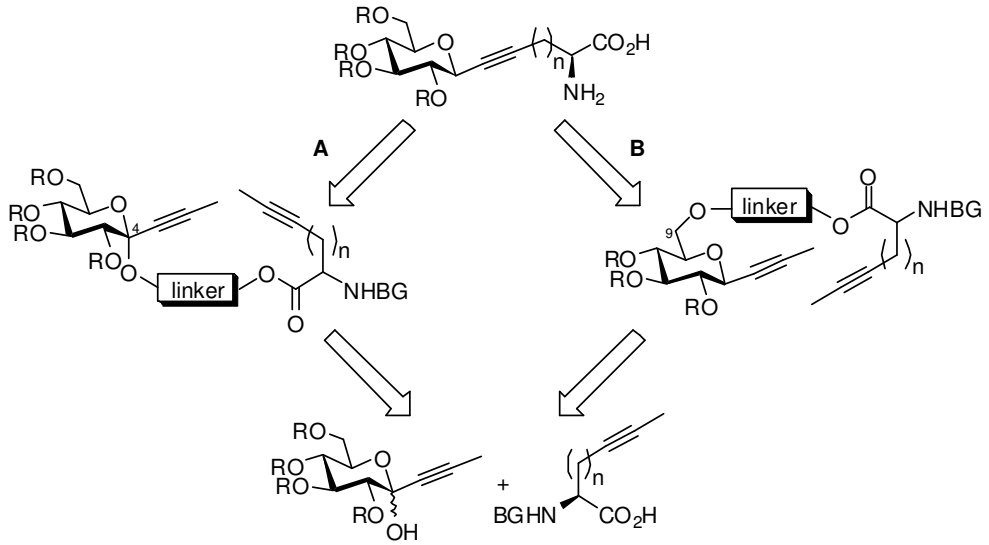
Schema 1 Isostere vervanging van de natuurlijke binding in glycoaminozuren en peptiden door alkyn (**A**) en triazool (**B**) linkers, die beide gemaakt kunnen worden uit glycoacetylenen

Glycoacetylenen, koolhydraten gefunctionaliseerd met een acetyleen groep aan één van de ringkoolstoffen, vertegenwoordigen een klasse van verbindingen die gebruikt kunnen worden als startpunt voor de synthese van diverse *C*-glycosiden zoals *C*-glycoaminozuren en peptiden. Het onderzoek in dit proefschrift richt zich op het gebruik van glycoacetylenen voor de synthese van verschillende klassen van stabiele glycopeptide mimetica en het bestuderen van de biologische eigenschappen van enkele daarvan.

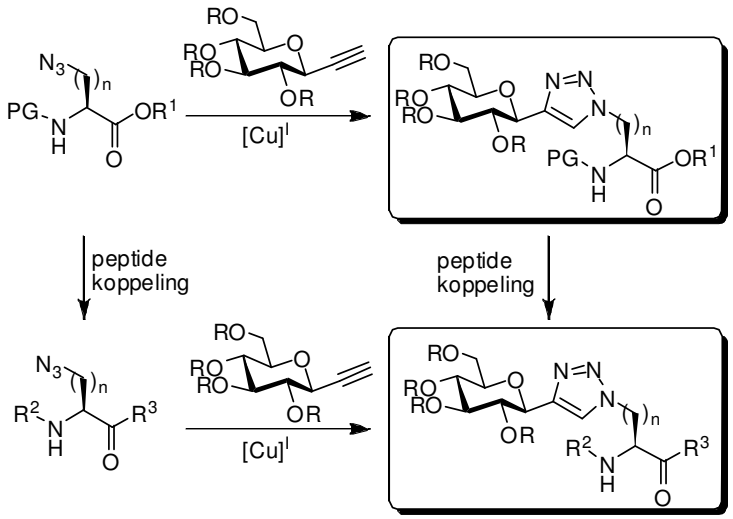
Hoofdstuk 1 geeft een literatuuroverzicht van verschillende methoden voor de synthese van glycoacetylenen. Meestal betreft dit de toepassing van een nucleofiele additie van een metaalacetylide op een suikerelectrofiel, in het bijzonder op het anomere koolstofatoom. Een geraffineerde keuze van condities en metaalionen (waaronder magnesium, lithium, zink en tin) leidt selectief tot vorming van het α- of

β-glycoacetyleen. Glycoacetylenen kunnen op verschillende manieren worden toegepast in de synthese van *C*-glycoaminozuren. Voorbeelden zijn onder meer alkaan-, alkyn-, tryptofaan-, en triazool-gelinkte glycoaminozuren, alsmede conformationeel vastgelegde glycoaminozuren.

In Hoofdstuk 2 is de synthese van alkyn-gelinkte glycoaminozuren via ringsluitingsalkynmetathese (RCAM) onderzocht. Twee verschillende strategieën zijn beschreven om alkynylaminozuren te koppelen met alkynylsuikers, nl. via een linker aan O-4 (Schema 2, optie **A**) of aan O-9 (optie **B**) van de alkynylsuiker. RCAM van de O-4 gelinkte diyn leidde niet tot een succesvol resultaat, maar gelukkig gaf toepassing van dezelfde condities op de O-9 gelinkte diynen wel de overeenkomstige alkyn-gelinkte glycoaminozuren van verschillende ketenlengte.



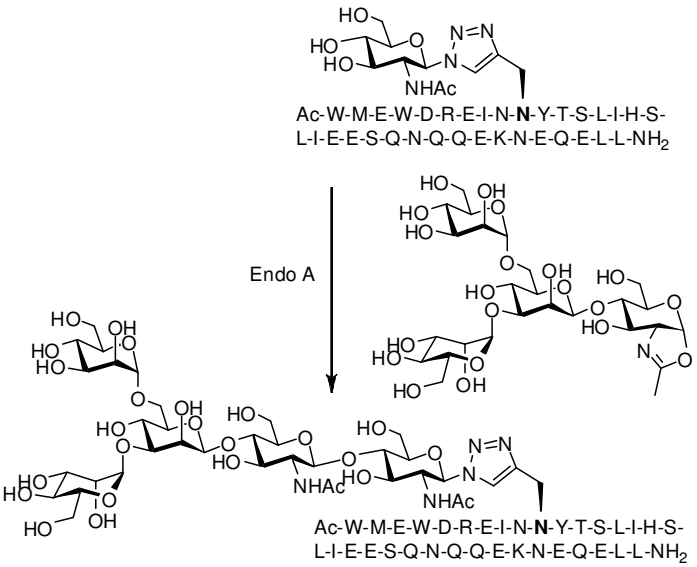
Schema 2 Retrosynthese van alkyn-gelinkte glycoaminozuren middels ringsluitingsalkynmetathese via een O-4 linker (optie **A**) of een O-9 linker (optie **B**)



Schema 3 Synthese van triazool-gelinkte glycoaminozuren en peptiden

In Hoofdstuk 3 is methodologie beschreven voor de synthese van triazool-gelinkte glycoaminozuren en peptiden gebaseerd op de koper(I)-gekatalyseerde azide acetyleen cycloadditie (CuAAC) van glycoacetylenen met azidoaminozuren (Schema 3). De resulterende triazolyl glycoaminozuren blijken geschikte bouwstenen te zijn voor de bereiding van de overeenkomstige grotere glycopeptiden. Een alternatieve route voor de synthese van deze triazolyl glycopeptiden verloopt via latere introductie van het koolhydraatfragment middels dezelfde cycloadditiereactie van glycoacetylenen met in dit geval azidopeptiden.

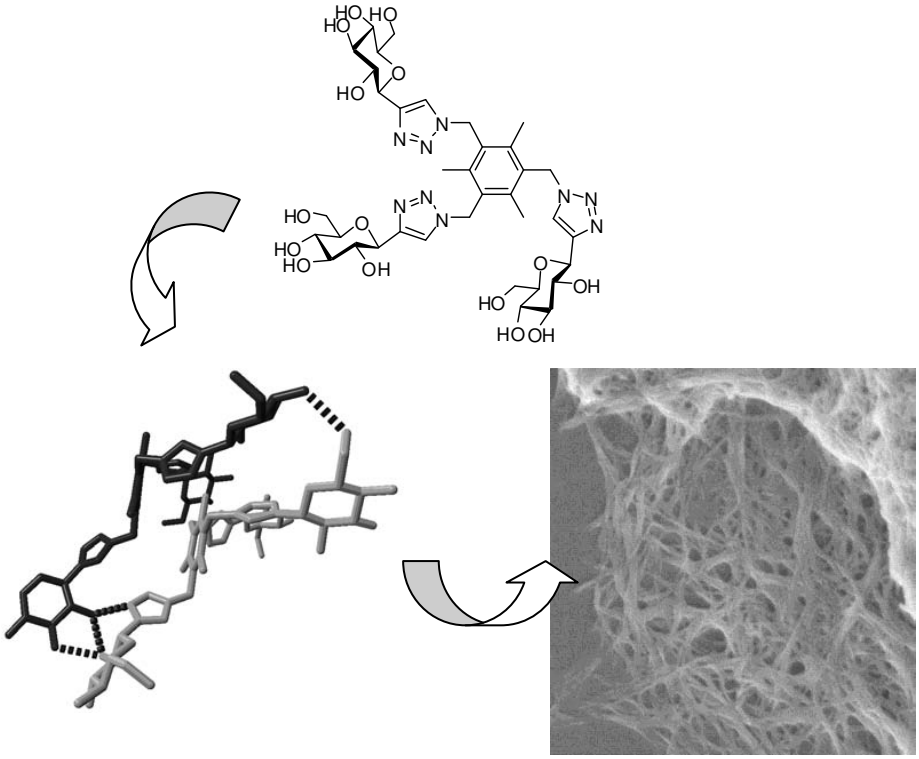
Hoofdstuk 4 beschrijft de chemoenzymatische transglycosylering van *C*- en *N*-triazool-gelinkte glycopeptiden met een oxazoline-geactiveerd koolhydraat onder invloed van het enzym Endo A (Schema 4). Terwijl de conversie van een *C*-triazolyl glycodipeptide stopte bij 50%, werd het vergelijkbare *N*-triazolylderivaat omgezet naar het overeenkomstige pentasaccharide met een efficiëntie van 80%. Dezelfde chemoenzymatische glycosyleringsprocedure werd ook succesvol toegepast op een meer complex glycopeptide, het triazool-gelinkte *N*-GlcNAc-gemodificeerde peptide C34, dat voorkomt in het *C*-terminale fragment van HIV-1 gp41. Het resulterende nieuwe triazool-gelinkte glycopeptide bleek volledig stabiel in aanwezigheid van peptide *N*-glycanase (PNGase F), een serum peptidase dat het overeenkomstige natuurlijke amide-gelinkte glycopeptide efficiënt hydrolyseert.



Schema 4 Endo A-gekatalyseerde glycosylering van GlcNAc-C34*

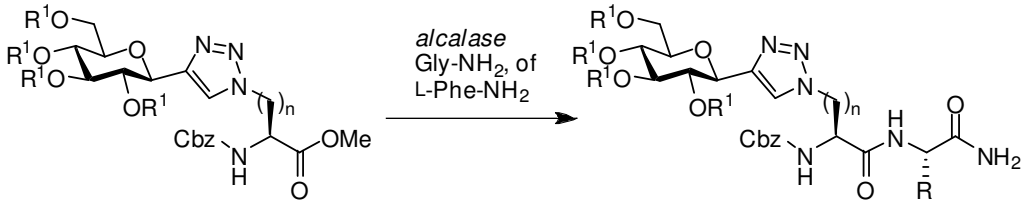
In Hoofdstuk 5 is de synthese van oligosacchariden door condensatie van glycoacetylenen met tri- en hexaazidohexamethylbenzeen in de aanwezigheid van koperdraad beschreven. Zoals weergegeven in Figuur 1, bleken de gevormde triazool-gelinkte oligosacchariden supramoleculaire gels te vormen, waarschijnlijk door een combinatie van waterstofbruggen tussen de saccharide fragmenten en π-π stacking van de areen eenheden, zoals blijkt uit berekeningen. De trisaccharide gel

werd gekarakteriseerd met de technieken SEM, TEM en AFM, die allen de aanwezigheid van verstrengelde fibers bevestigden.



Figuur 1 Triazool-gelinkte trisaccharide aggregatie leidt tot tot supramoleculaire gels

Chemoenzymatische peptidekoppeling van azide- en acetyleen-bevattende aminozure methylesters met natuurlijke aminozuuramiden onder invloed van het enzym *alcalase* is onderzocht in Hoofdstuk 6 (Schema 5). Deze condensatie bleek heel schoon te verlopen en in hoge opbrengst. De inbouw van glycoaminozuren in peptiden werd voor verschillende beschermgroepen en zijketens onderzocht en bleek het meest succesvol voor glycoaminozure esters met een acetyl beschermgroep (R^1 = Ac) in combinatie met een korte zijketen (n = 1).



Schema 5 Chemoenzymatische koppeling van glycoaminozure esters met glycine amide en phenylalanine amide

Dankwoord

Dan nu eindelijk het gedeelte van het boekje wat niet meer onder invloed van zeer grote hoeveelheden koffie geschreven hoeft te worden, maar waar ik in alle rust aan kan werken onder het genot van niets minder dan een heerlijk koud glas bier!

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I would like to thank prof. Lai-Xi Wang and Dr. Wei Huang, from the University of Maryland, for their chemoenzyamtic glycosylation reactions and the stability testing's, I hope that the good results will be published soon, and that this research will lead to more interesting results in the future.

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Terwijl ik dit zo schrijf bedenk ik mij dat heel veel vriendschappen zijn ontstaan met mensen waarmee ik het lab heb mogen delen, daaruit trek ik dan ook de conclusie dat op het lab dus niet alleen chemische preparaten worden gesynthetiseerd maar ook goede vriendschappen! Van deze vriendschappen wil ik er toch twee in het bijzonder noemen, en wel mijn paranimfen Sander en Richard.

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Allen, hef het glas en Proost!

Stan

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Curriculum Vitae

Stan Groothuys werd geboren op 10 januari 1978 te Hengelo. Na het behalen van het HAVO diploma aan De Grundel te Hengelo, startte hij in 1996 met de opleiding HBO Chemie aan de Saxion Hogeschool Enschede. In 2000 studeerde Stan af in de richting Organische Chemie na zijn afstudeerstage bij DSM Research te Geleen. Aansluitend begon de auteur aan de studie Scheikunde aan de toenmalige Katholieke Universiteit Nijmegen, die hij in 2003 voltooide met het doctoraal examen. De hoofdvakstage werd in de vakgroep Synthetisch Organische Chemie van prof. dr. F.P.J.T. Rutjes uitgevoerd onder de leiding van dr. F. L. van Delft. Van maart 2003 tot maart 2007 was hij aangesteld als junior onderzoeker in dezelfde vakgroep aan de Radboud Universiteit Nijmegen. De resultaten van het onderzoek staan in dit proefschrift beschreven en zijn uitgevoerd onder leiding van prof. dr. F.P.J.T. Rutjes en dr. F.L. van Delft. Sinds 1 november 2007 is Stan werkzaam als junior project manager bij Schering-Plough te Oss.